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The effect of thermogenic stimuli on the immune system
Vliv termogenních podnětů na imunitní systém

Doctoral thesis

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Prague, 2025

Author´s declaration

I declare that this thesis has been composed entirely by myself and that all information sources and literature have been properly cited. This thesis has not been submitted, in whole or in part, an any previous application for a degree. Artificial intelligence (Writefull version 2025.8.0 and DeepL version 25.1.6. 15223+) was used as grammar editors of the text.

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Abstract

Organisms possess sophisticated defense mechanisms to maintain homeostasis and combat threats. The immune system plays a pivotal role in this defense, recognizing and responding to threats from both the internal and external environments. While the influence of various factors on the immune system is well recognized, the effect of temperature remains an area of significant interest. The complex interplay between thermogenic stimuli and immune responses is explored in this dissertation.

Initially, we investigated the effects of high temperature-dependent TRPV1 channel activation in macrophages. The results obtained demonstrate that TRPV1 activation by capsaicin in an inflammatory environment induces a phenotypic shift in macrophages from pro-inflammatory M1 to anti-inflammatory M2b-like. This transition is accompanied by alterations in cytokine production, up-regulation of co-stimulatory molecules, and enhanced T cell proliferation.

Secondly, we investigated the effects of chronic cold exposure on the immune system in a rat model. Our observations revealed substantial changes in the immune system during the process of cold acclimatization, including an increase in the abundance of γδ T cells in various tissues. These findings suggest a critical role for γδ T cells in the regulation of thermogenesis. Furthermore, we observed that cold exposure altered the immune response to TLR2 and TLR4 stimulation, highlighting the profound impact of temperature on immune function. Finally, we translated some of these findings to human subjects. Specifically, we observed an increase in the population of γδ T cells in the blood of volunteers who regularly swim in cold water, in comparison to a control group.

This dissertation offers novel insights into the intricate relationship between thermogenic stimuli and the immune system. The findings underscore the potential for targeting TRPV1 and γδ T cells to modulate immune responses. The implications of these findings are significant, as they offer novel perspectives on comprehending and potentially manipulating the immune system under various physiological and pathological conditions.

Abstrakt (CZ)

Organismy mají důmyslné obranné mechanismy, které jim umožňují udržovat homeostázu a bojovat proti hrozbám. Klíčovou roli v této obraně hraje imunitní systém, který rozpoznává hrozby z vnitřního i vnějšího prostředí a reaguje na ně. Zatímco vliv různých faktorů na imunitní systém je dobře znám, vliv teploty zůstává oblastí značného zájmu. Tato disertační práce zkoumá složitou souhrou mezi termogenními podněty a imunitními reakcemi.

Nejprve jsme zkoumali účinky aktivace kanálu TRPV1 v makrofázích v závislosti na vysoké teplotě. Získané výsledky ukazují, že aktivace TRPV1 kapsaicinem v zánětlivém prostředí vyvolává fenotypový posun makrofágů z prozánětlivého M1 na protizánětlivý M2b-like. Tento přechod je doprovázen změnami v produkci cytokinů, upregulací ko-stimulačních molekul a zvýšenou proliferací T-buněk.

Za druhé jsme zkoumali účinky chronické expozice chladu na imunitní systém na modelu potkana. Naše pozorování odhalila podstatné změny v imunitním systému během procesu aklimatizace na chlad, včetně zvýšení množství γδ T buněk v různých tkáních. Tato zjištění naznačují, že γδ T buňky hrají zásadní roli v regulaci termogeneze. Dále jsme pozorovali, že vystavení chladu změnilo imunitní odpověď na stimulaci TLR2 a TLR4, což zdůrazňuje hluboký vliv teploty na imunitní funkce. Nakonec jsme některá z těchto zjištění převedli na lidské subjekty. Konkrétně jsme pozorovali zvýšení populace γδ T buněk v krvi jedinců, kteří pravidelně plavou ve studené vodě, ve srovnání s kontrolní skupinou.

Tato disertační práce nabízí nový pohled na složitý vztah mezi termogenními podněty a imunitním systémem. Zjištění podtrhují potenciál cílení na TRPV1 a γδ T buňky za účelem modulace imunitních reakcí. Důsledky těchto zjištění jsou významné, protože nabízejí nové perspektivy pro pochopení a potenciální manipulaci s imunitním systémem v různých fyziologických a patologických stavech.

Table of contents

ABSTRACT 5

ABSTRAKT (CZ) 6

LIST OF ABBREVIATIONS 9

INTRODUCTION 12

Thermoregulation 14

Thermogenesis 15

Fever and pathophysiological conditions 19

TRP channels 19

TRPM 20

TRPV 21

Macrophages 23

M1 macrophages 24

M2 macrophages 24

TRPV1 on immune cells 26

TRPV1 on macrophages 27

Cold adaptation 28

The immune system and the cold temperature 29

Immune cells involved in cold adaptation 31

Inflammation 34

Pattern recognition receptors (PRRs) 35

Toll-like receptors (TLRs) 35

AIMS 40

RESULTS 42

I. Lipopolysaccharide pretreatment increases the sensitivity of the TRPV1 channel and promotes an anti-inflammatory phenotype of capsaicin-activated macrophages 43

Discussion I 71

II. Immune response to cold exposure: Role of γδ T cells and TLR2-mediated inflammation 76

Discussion II 105

FINAL DISCUSSION 109

CONCLUSIONS..... 113
REFERENCES..... 115

List of abbreviations

AR	adrenergic receptor
Arg-1	arginase 1
ATP	adenosine triphosphate
BAT	brown adipose tissue
CCL	chemokine C-C motif ligand
CLR	C-type lectin receptor
CNS	central nervous system
COX-2	cyclooxygenase 2
CXCL	chemokine C-X-C motif ligand
DAMP	damage/danger-associated molecular pattern
DC	dendritic cell
FGF-21	fibroblast growth factor 2
HSP	heat shock protein
CHIKV	Chikungunya virus
IF	impact factor
IFN	interferon
IFNγ	interferon gamma
IL	interleukin
IL-1R	interleukin 1 receptor
ILC	innate lymphoid cell
iNKT	invariant natural killer T cell
iNOS	inducible nitric oxide synthase
IRF	interferon regulatory factor
KO	knockout
LPBd	dorsal segment of the lateral parabrachial nucleus

LPBel	external segment of the lateral parabrachial nucleus
LPS	lipopolysaccharide
MAP	mitogen-activated protein
MerTK	mer receptor tyrosine kinase
MSC	mesenchymal stem cell
MyD88	myeloid differentiation primary response 88
NE	norepinephrine
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor-kappa-light-chain-enhancer of activated B cells
NLR	NOD-like receptor
NO	nitric oxide
PAMP	pathogen-associated molecular pattern
PGC1-α	PPARγ co-activator-1 α
PNS	peripheral nervous system
POA	preoptic area
PPAR	peroxisome proliferator-activated receptor
PRR	pattern recognition receptor
RLR	RIG-I-like receptor
ROS	reactive oxygen species
SAM	sympathetic neuron-associated macrophage
TAM	tumor-associated macrophage
TCR	T cell receptor
TGF-β	transforming growth factor-β
Th cell	T helper cell
TLR	toll-like receptor
TNFα	tumor necrosis factor alpha
Treg	regulatory T cell
TRIF	TIR-domain-containing adapter-inducing interferon-β

TRP channel	transient receptor potential channel
TRPA	ankyrin TRP
TRPC	canonical TRP
TRPM	melastatin-related TRP
TRPML	mucolipin TRP
TRPN	no mechanoreceptor potential C TRP
TRPP	polycystic TRP
TRPV	vanilloid TRP
UCP1	uncoupling protein 1
VR1	vanilloid receptor 1
WAT	white adipose tissue
γδ T cell	gamma delta T cell

Introduction

Throughout its lifetime, an organism is required to manage a series of events that may pose a threat to its integrity. Consequently, it has evolved multiple defense mechanisms to protect the body. The objective is to preserve homeostasis, which is crucial to maintaining vital metabolic functions and processes that occur within every living cell. The primary system that organisms have evolved to maintain integrity and homeostasis is the immune system. The immune system's role is to detect potential threats originating from either external or internal sources and to initiate an immune response that will ensure the survival of the organism. Nonetheless, the immune system is susceptible to various factors that can alter its functionality and efficiency. Such influences can manifest as either beneficial or detrimental. Influencing factors include stress, dietary habits, physical activity, lifestyle choices, adequate sleep, and age, in addition to various diseases, injuries, infections, or subsequent treatments. Among environmental factors, temperature is a well-established modulator of immune function and overall health.

It is imperative to preserve all physiological functions of the organism, regardless of elevated or reduced ambient or internal temperatures. The persistence of fundamental metabolic reactions is crucial for maintaining a stable body temperature, with the involvement of almost all physiological systems in this process. Under circumstances characterized by extreme changes, some physiological processes may be overlooked. However, it is necessary for the immune system to maintain its functionality, as failure of this system could have serious consequences. It is widely acknowledged that variations in temperature have a significant impact on the immune system. Immune responses and processes function differently compared to optimal conditions, resulting in altered immune reactions. Consequently, the recognition of threats and the combat against infections may vary based on ambient temperature, affecting their efficacy accordingly.

Nevertheless, the immune system actively participates in regulatory processes that respond to temperature variations and serves as a crucial component in adaptation mechanisms. In the absence of a fully functioning immune system, the maintenance of fundamental metabolic processes in extremes of cold or heat becomes extremely challenging, thus significantly increasing vulnerability to infections and disrupting homeostasis. While the immune system continues to operate effectively under altered temperature conditions, it must also actively engage in the physiological adaptation to novel temperature settings and guarantee the optimal performance of other bodily systems.

Consequently, the role of the immune system under varying temperature conditions is of critical importance. Notably, numerous studies have focused on the effects on the organism when exposed to thermal stimuli, yet relatively few have specifically addressed the immune system's responses. In this dissertation, I intend to consolidate the knowledge acquired throughout my doctoral research on the impact of thermogenic stimuli on the immune

system. Comprehending the intricate interconnections between ambient temperature, its discernment, and subsequent initiation of appropriate thermoregulatory mechanisms by the immune system, in addition to maintaining its fundamental operations, presents a highly promising therapeutic objective. By employing ambient temperature or targeting specific temperature receptors, it is possible to modulate immune system functions and efficacy, as well as activate metabolic pathways involved in the thermoregulation process. Consequently, these applications hold substantial potential for clinical practice and medical advancement.

Thermoregulation

Thermoregulation is defined as a process of regulating the physiological body temperature through the equilibrium between heat generation and dissipation. In the context of humans, the term "normothermic" is used to denote an individual whose body temperature is maintained within a range of 37°C +/- 0.5°C. In contrast, rats exhibit a slightly elevated temperature of 37.3 °C +/- 0.5 °C, while mice present a lower temperature of 36.5 °C +/- 0.5 °C [1]. Maintaining an appropriate temperature range is essential to maintain homeostasis and ensure optimal metabolic function [2]. The peripheral thermoreceptors, located in the skin, are responsible for detecting surface temperatures, while the central thermoreceptors, located in the viscera, spinal cord, and hypothalamus, sense the core temperature. The thermoregulatory center is located in the hypothalamus, more specifically in the preoptic area (POA). POA integrates peripheral and central thermosensory neural inputs and transmits signals to effector organs to initiate involuntary thermoregulatory responses [3],[4]. Neurons that are sensitive to elevated temperatures are found in the dorsal segment of the lateral parabrachial nucleus (LPBd), and their activation leads to vasodilation and suppression of thermogenesis. Conversely, neurons that are sensitive to cold are located in the external segment of the lateral parabrachial nucleus (LPBel), and upon activation, they induce vasoconstriction and promote thermogenesis (Fig. 1) [4],[5].

The culmination of the physiological response to elevated body temperature is the dissipation of heat through several mechanisms. Among these mechanisms, there is an increase in sweating, facilitated by the activation of sympathetic cholinergic fibers that innervate the sweat glands. Concurrently, there is suppression of sympathetic activity in cutaneous blood vessels, enhancing blood flow to the skin and thereby increasing heat loss. Furthermore, a decrease in the release of catecholamines from the adrenal glands and thyroid hormones from the hypothalamus results in a reduced metabolic rate. Behavioral adaptations, such as decreased physical activity, the adoption of an "open" body posture, and a reduction in appetite, also occur [5],[6].

Responses to decreased body temperature are correlated with mechanisms that generate heat. The sympathetic nervous system induces vasoconstriction of skin arterioles, thereby reducing cutaneous blood flow and subsequently reducing heat dissipation. The secretion of thyroid hormones, along with the enhanced synthesis of catecholamines by the adrenal glands, helps to elevate the metabolic rate. Moreover, behavioral adaptations, including increased propensity for movement, the adoption of a "closed" body posture, and heightened appetitive drive, further contribute to the regulation of body temperature. Furthermore, the process of thermogenesis is initiated [5],[6].

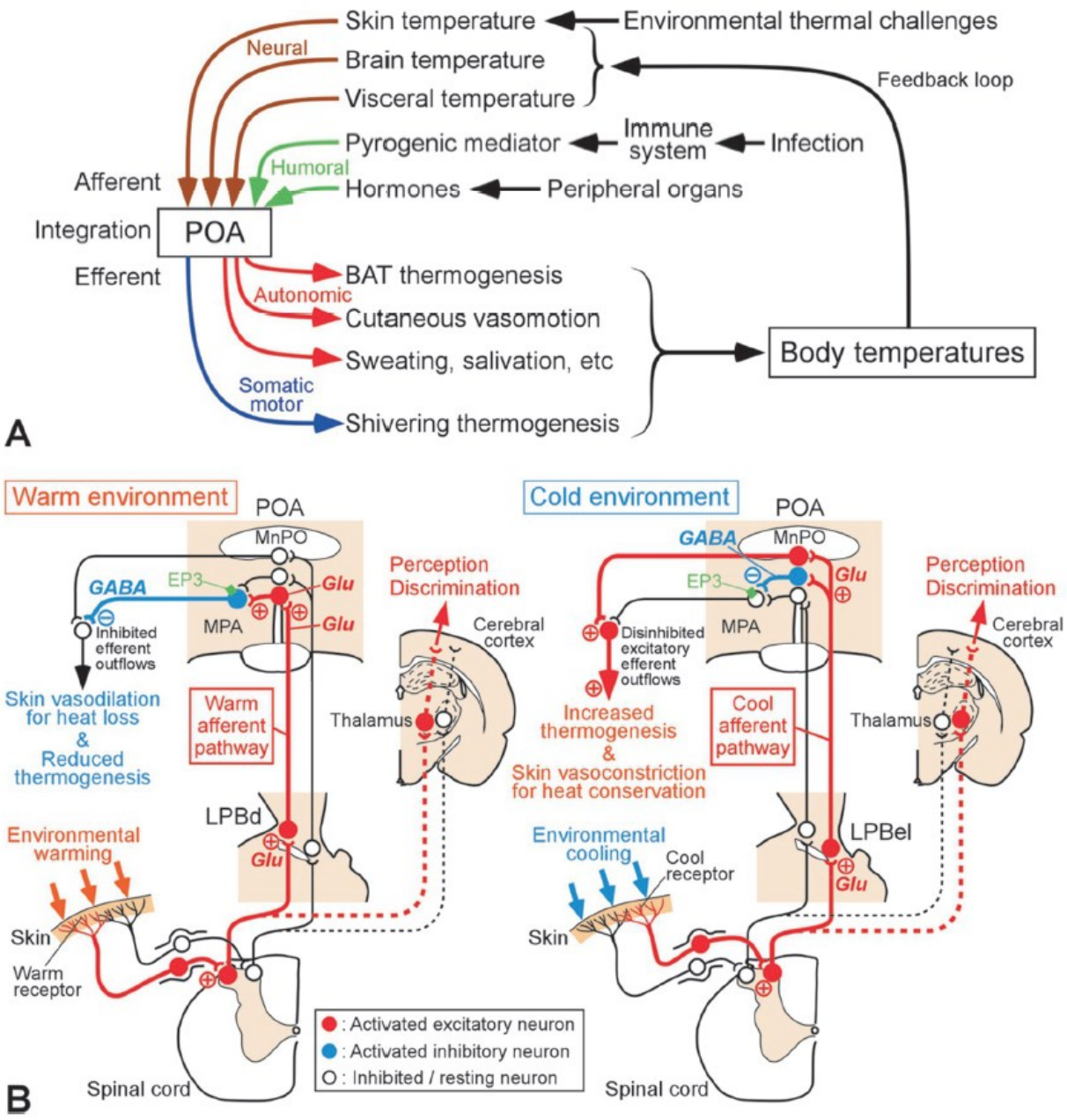


Figure 1 (A) *Simplified diagram of the thermoregulatory system. The preoptic area (POA) receives neural (brown) and humoral (green) afferents to provide thermoregulatory efferent signals.* ***(B)*** *Schematic model of central thermoregulatory pathways in warm and cold environments. See text for details. BAT, brown adipose tissue; GABA, gamma-aminobutyric acid; Glu, glutamate; LPBd, dorsal part of the lateral parabrachial nucleus; LPBel, external part of the lateral parabrachial nucleus; MnPO, median preoptic nucleus; MPA, medial preoptic area. Taken from Nakamura K., 2018* ***[4]***

Thermogenesis

Thermogenesis is defined as the process by which an organism produces heat actively. Two distinct categories of thermogenesis have been identified: shivering and non-shivering thermogenesis. These categories diverge based on the underlying mechanisms and the

specific tissue responsible for heat production. In the subsequent paragraph, a comprehensive overview of both types will be provided, with a more in-depth examination of non-shivering thermogenesis, the model of which was used in the experimental part of this dissertation.

Shivering thermogenesis

Shivering thermogenesis is a process that utilizes striated muscle to generate heat. It is triggered by the stimulation of cold receptors located on the skin, which in turn activates the alpha and gamma moto neurons. This activation leads to the spontaneous, rapid, and repeated contraction of muscles comprising fibers I and II. This process is driven by the conversion of adenosine triphosphate (ATP) into kinetic energy without the expenditure of work, which is subsequently released as heat. In large mammals, including humans, shivering thermogenesis is the predominant mechanism of heat generation in response to cold exposure [5],[7].

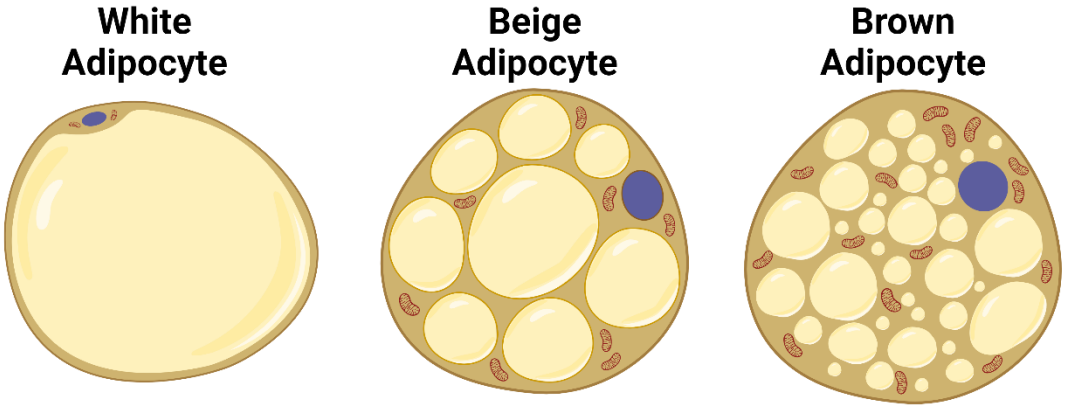
Non-shivering thermogenesis and brown adipose tissue

Non-shivering thermogenesis is a process that occurs in brown adipose tissue (BAT), a specialized fat depot distinct from white adipose tissue (WAT), which is primarily responsible for energy storage. Typically, adipocytes of WAT contain a single large fat droplet, a compressed nucleus close to the cell membrane, and a smaller number of thin and elongated mitochondria [8]–[10]. In contrast, BAT adipocytes are characterized by multilocular fat droplets and a nucleus that is located more centrally within the cell. A pronounced distinction emerges in the quantity of mitochondria in BAT adipocytes, which are often more substantial in size and possess a greater number of cristae. The brown color of BAT is associated with a higher concentration of mitochondrial cytochrome and stronger vascularization compared to WAT [11]. The thermogenic function of BAT is driven by the expression of uncoupling protein 1 (UCP1), also known as thermogenin in some studies, on the inner mitochondrial membrane (Fig. 2) [9].

Cold exposure and subsequent activation of cold receptors trigger activation of BAT through the release of norepinephrine (NE) from the sympathetic nervous system of the adrenal glands. This sympathetic activation stimulates the metabolic pathway, leading to the breakdown of triacylglycerols and the subsequent formation of free fatty acids. The subsequent β-oxidation of these fatty acids produces acetyl coenzyme A, which enters the citrate cycle, where a sequence of biochemical reactions leads to the formation of a proton gradient in the inner membrane of mitochondria in the final respiratory chain. However, in adipocytes of BAT, NE binds to the β3adrenergic receptor (AR), inducing the expression of UCP1. This protein has the ability to uncouple the proton gradient, a process in which ATP synthesis and the return of protons via ATP-synthase do not occur. Instead, protons pass directly back into the mitochondrial matrix. This results in the dissipation of the proton motive force, which is converted into heat [12]. BAT is primarily responsible for thermogenesis in small mammals, young animals, and hibernating animals [13]. In humans, BAT is active in newborns and is found in the interscapular region; however, it regresses with age [8]. For a

considerable period, it was believed that BAT disappeared entirely in adults. It was not until the development of positron emission tomography/computed tomography, which is used in the diagnosis of malignancies, that the presence of BAT in adult patients was unexpectedly revealed [14]. The currently accepted localization of BAT in adults is in the cervical, supraclavicular, paravertebral, periaortic, and perirenal depots [8].

A



B

	Mitochondrial density	UCP1 expression	LD Morphology	Primary funciton
White	*Low*	-	*Uni-locular*	*Energy storage Endocrine*
Beige	*Medium*	*+*	*Multi-locular*	*Thermogenesis Endocrine*
Brown	*High*	*+++*	*Multi-locular*	*Thermogenesis Endocrine*

C

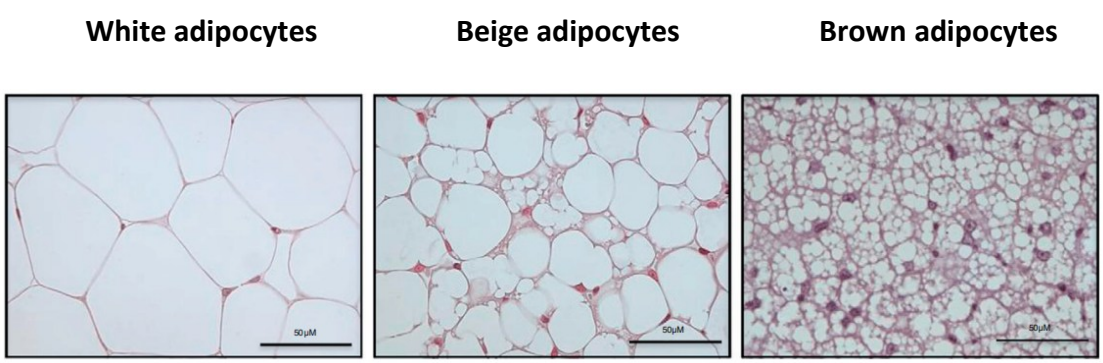


Fig. 2 (A) *Adipocytes characteristic of individual types of adipose tissue. Adipocyte originating from white adipose tissue (left), beige adipose tissue (middle), and brown adipose tissue (right). Fat droplets (yellow), nucleus (blue) and mitochondria (red) are depicted.* ***(B)*** *Brown adipocytes contain many UCP1 positive mitochondria, numerous small lipid droplets and are involved in thermogenesis. In contrast, white adipocytes contain few mitochondria that do not express UCP1 and a single, large lipid droplet for triglyceride storage. Beige adipocytes have an intermediate phenotype between classic brown and white adipocytes. Taken and adapted from Jung et al., 2018; Suchacki and Stimson, 2021 [8].* ***(C)*** *Morphology of three different types of adipocytes. Hematoxylin and eosin staining of white (left), beige (middle) and brown (right) adipocytes. Scale bars 50 µm. Taken and adapted from Keipert and Jastroch, 2014 [10]*

In addition to classic BAT adipocytes, the presence of beige adipocytes has been documented. These cells are derived from WAT precursors or through direct redifferentiation of white adipocytes. Morphologically, these cells exhibit intermediate characteristics between BAT and WAT. However, they are characterized by induced expression of UCP1. This process is referred to as "beiging" of adipose tissue. These beige adipocytes are utilized in adults, who, in contrast to small mammals, are unable to form new brown adipocytes. The beiging process is reversible; however, if the organism remains in chronic cold exposure for an extended period, beige adipocytes can adopt their new phenotype, thus fulfilling the function of BAT [8],[15].

It has been shown that the amount and activity of BAT are also influenced by various factors other than the reduced temperature. One such factor may be the composition of food, such as the presence of fatty acids or thermosensor ligands, which contribute to or inhibit the adipose tissue beiging and activation. For instance, omega-3 fatty acids have been observed to enhance the expression of UCP1 and β3-AR [16]–[18]. The catechins and caffeine present in tea have been observed to activate BAT in research models[19],[20]. The activation of BAT can also be stimulated by curcumin, a component of the spice turmeric [21], as well as by components found in garlic [22] or ginger extracts [23]. Additional components affecting BAT include thermosensor activators from the transient receptor potential (TRP) family, such as menthol or capsaicin. These channels will be discussed in subsequent chapters.

Another factor to consider is physical activity. Physical exercise has been shown to increase body temperature, activate the sympathetic nervous system, and enhance blood flow through skeletal muscles. Subsequent increase in NE concentration leads to an increase in the number of thermogenic adipocytes and UCP1 activation [24]. However, contradictory studies have also been published, reporting a decrease in UCP1 activity after exercise [25],[26]. Nevertheless, there is an agreement on the influence of myokines and factors produced by muscle tissue during exercise, which can increase thermogenesis. These factors include IL-6, IL-39, or irisin, which activate the transcription factor peroxisome proliferator-activated receptor α (PPAR-α) and its co-activator-1 α (PGC1-α). These factors are then responsible for mitochondrial biogenesis and thermogenesis [27]–[30]. The final noteworthy regulator is the microbiome, which undergoes substantial alterations when the organism is exposed to cold. This change is

associated with an increase in metabolic activity and a beiging of WAT. In germ-free mice, thermogenic capacity is reduced and UCP1 expression is decreased [31].

Fever and pathophysiological conditions

Fever is characterized by an elevation in body temperature above the normal range, regulated by the POA within the thermoregulatory center. Its etiology is multifactorial, with different origins, including infection, inflammation, autoimmune disorders, or medication effects. It is not merely transient overheating but is induced by the activity of pyrogens. Exogenous pyrogens originate externally and trigger the production of endogenous pyrogens, specifically pro-inflammatory cytokines including interleukin 1 (IL-1), IL-6, tumor necrosis factor alpha (TNFα), and interferon gamma (IFNγ). These cytokines induce the synthesis of prostaglandin E2 through cyclooxygenase 2 (COX-2), thereby stimulating the production of thermogenic neurotransmitters, which culminates in an increase in body temperature. Fever plays a crucial role in host defense; it enhances leukocyte mobility and proliferation, increases phagocytic capabilities, and mitigates the effects of endotoxins. Consequently, pharmacological intervention to suppress fever is usually avoided unless the fever is excessively high, persists for an extended period, or poses a threat to the patient's life [32].

Pathological conditions affecting thermoregulation pose significant health risks by increasing susceptibility to temperature extremes. These are often different types of neuropathies, endocrinological disorders, or problems with insufficient sweating. This can be observed, for example, in patients with Sjögren's syndrome, multiple sclerosis, or Parkinson's disease [33]–[35]. Thermoregulatory disorders are also a complication in people with spinal cord or brain injuries, as the connection to the hypothalamus is often damaged. Another complication is the loss of physical sensation of the skin, making it impossible to detect hot or cold stimuli [36].

TRP channels

Thermoreceptors play a critical role in sensing external temperatures. Notable thermoreceptors include the family of TRP channels, a subject of particular interest in this research and this doctoral thesis. These channels are characterized by their high degree of conservation across species, with expression observed in both invertebrates and vertebrates. Notably, a homologous channel, designated as Yvc1, has been observed to be expressed in yeast [37]. However, the expression of these channels in plant cells remains to be confirmed. The TRP family was first identified in 1969 in the organism *Drosophila melanogaster*, which initiated extensive research in this area. Within a relatively brief period, six subfamilies were defined: canonical TRP (TRPC), melastatin-related TRP (TRPM), ankyrin TRP (TRPA), mucolipin TRP (TRPML), polycystic TRP (TRPP), and vanilloid TRP (TRPV) [38]–[41]. The seventh subfamily named no mechanoreceptor potential C (TRPN) was only identified in 2000 [42] and its complete structure remained unresolved until 2017 [43].

Each TRP cation channel is comprised of six transmembrane helices, and the passage pore is typically situated between the fifth and sixth domains. The protein termini are oriented towards the cytosolic side of the membrane, while the N terminus is specifically adapted for binding modulators [44],[45]. However, individual subfamilies display a wide variety of gating mechanisms and consequently functions, resulting in a diverse array of final structures [40].

The TRPN family has been described as mechanoreceptors [46],[47]. The TRPP subfamily, which is found mainly in renal cilia, is also included in the category of mechanoreceptors. Autosomal dominant polycystic kidney disease has been linked to a mutation in genes that encode these proteins [48]. The precise function of the TRPML family remains to be elucidated. However, one of its members, the TRPML1 protein, is localized in endosomes, where it participates in proton transport [49]. Furthermore, its activity is also associated with autophagosome biogenesis [50]. The TRPA subfamily is represented solely by TRPA1. It is a significant protein due to its role in the transmission of painful stimuli and neurogenic inflammation. Activators of this protein include pungent oils such as those found in mustard or garlic [51]. The TPRC group was discovered as the very first subfamily. Because it was discovered mainly due to its molecular identity, its properties remained in the background for a long time. TRPCs are expressed in many tissues, including the brain, testis, or heart. We now know that its function is primarily related to calcium cation transport and regulation by the phospholipase C pathway [52]. The thermoreceptors that have been identified to date are classified within the TRPM and TRPV families.

TRPM

The TRPM subfamily comprises eight distinct channels, designated as TRPM1-8, which exhibit various functional characteristics. It is hypothesized that functional channels form tetramers and that individual proteins differ significantly structurally from other TRP channels [53]. While TRPM4 has been implicated in cardiac conduction [54], TRPM5 has been associated with taste recognition [55], and TRPM6 plays a role in kidney and intestinal magnesium absorption [56], TRPM2, TRPM3, and TRPM8 channels are involved in response to temperature. TRPM3 is a Ca^{2+} permeable non-selective channel that is predominantly expressed in peripheral sensory neurons of the dorsal root ganglia. It is activated by high temperatures, as well as by inflammatory pain [57],[58]. TRPM2, on the other hand, functions as a hypothalamic heat sensor, where it can influence fever and hypothermia [59]. TRPM8, another non-selective cation channel, is expressed on sensory nerves and is known to be activated by cold temperatures. In addition, TRPM8 has been observed to be activated by plant-derived ligands, such as menthol or eucalyptol [60],[61]. This property contributes to the perception of cold sensations triggered by products or foods derived from mint or eucalyptus. TRPM8 is also involved in the perception of cold pain [60] and appears to be crucial for the detection of reduced temperature [62]. The role of TRPM8 is also described in the switch between fever and hypothermia [63]. Moreover, TRPM8 expression has been observed in mouse BAT adipocytes, and its activation by menthol has been shown to increase UCP1 expression [64].

Furthermore, menthol activation has been shown to increase UCP1 expression in human WAT, thereby aiding thermogenesis [65].

The immune response is also associated with signaling associated with TRPM8 activation. Activation of TRPM8 initiates a signaling pathway that results in increased blood pressure and increased plasma proteins concentration. This results in an increase in the concentration of immunoglobulins, which is associated with immunosuppression [66]. Furthermore, TRPM8 activation has been shown to negatively regulate the production of the pro-inflammatory cytokine TNFα [67]. In addition, TRPM8 stimulation in macrophages modulates the colitis model by altering the balance of TNFα and IL-10 production [68]. However, in T lymphocytes, activation of the TRPM8 channel has been shown to increase proliferation and promote the production of inflammatory cytokines [69]. Consequently, the precise mechanisms through which TRPM8 influences the immune system have not yet been elucidated.

TRPV

The TRPV channel subfamily has received the most research attention of all the TRP subfamilies. It is named after the well-known member, TRPV1, which is sensitive to vanilloids [70]. This subfamily comprises six distinct TRPV1-6 channels, which exhibit divergent Ca^{2+}/Na^{+} selectivity. These channels possess both homo and hetero-tetrameric structure [41],[71]. Notably, TRPV5 and TRPV6 exhibit no sensitivity to temperature changes, in contrast to the other TRPV1-4 channels, which are thermosensitive and thus termed "thermoTRPVs." These channels exhibit distinct differences in selectivity, associated subunits, and temperature sensitivity. All four are expressed in the central nervous system (CNS), but their expression differs in other tissues [41]. TRPV3 is expressed in keratinocytes [72], while TRPV4 is expressed in sperm [73]. However, the TRPV1 channel is the most well-known and studied, and since it is important for this thesis, the following section will focus on it in depth.

TRPV1

The TRPV1 receptor was first identified in 1997 by a research team led by David Julius and was initially referred to as vanilloid receptor 1 (VR1) [70]. For this groundbreaking discovery, David Julius was honored with the prestigious Nobel Prize in Physiology and Medicine in 2021. He shared this honor with Ardem Patapoutian, who identified other ion channels involved in sensory perception. TRPV1 is typically assembled as a homotetramer (Fig. 3A) [74], and the structure of individual monomers is indicated in Figure 3B [44]. The pore is formed by six transmembrane helices in conjunction with a loop. The extracellular portion of the channel is relatively wide, facilitating access [44],[75].

Initial research on TRPV1 focused on its expression in neurons [70], subsequently followed by an intensive investigation of its properties [76],[77]. In particular, the properties of TRPV1 associated with pain perception and nociception have been studied. These investigations have led to the identification of TRPV1 as an important therapeutic target in the field of analgesia [78]. It is expressed in the CNS in spinal and supraspinal structures, as well as in peripheral

primary afferent neurons and dorsal root ganglia in the peripheral nervous system (PNS) [79]. Recent findings have revealed that TRPV1 expression is not limited to the nervous system; rather, this protein is present in numerous types of tissues, including the lungs, skin, and gastrointestinal tract [80],[81]. Notably, its expression in the corneal endothelium [82], capillaries [83], and cerebromicrovascular endothelium [84] is of particular interest. Its presence in sperm [85] and epithelial cells [81],[86] has also been described. Furthermore, its expression has been documented in the immune system [87].

The presence of TRPV1 expression has been confirmed in a variety of immune cells, including T cells [88],[89], NK cells [90], neutrophils [90],[91], mast cells [92],[93], dendritic cells (DC) [94],[95], and macrophages [96],[97].

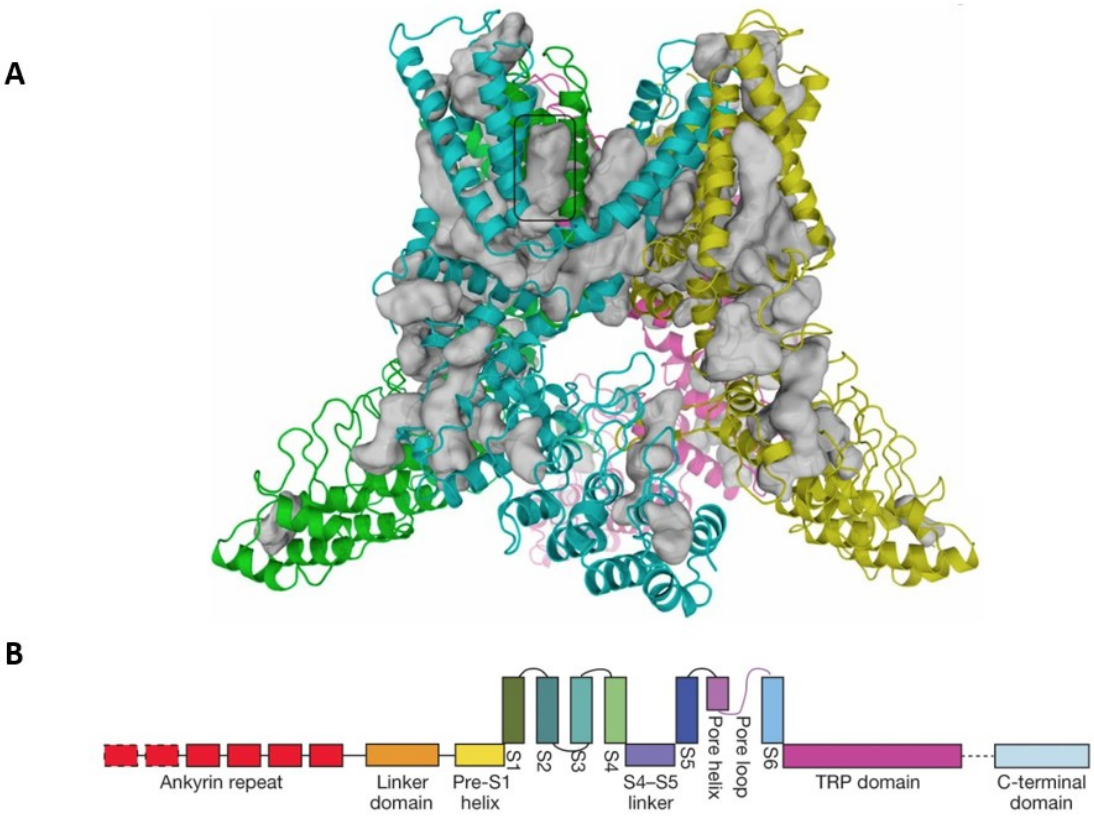


Fig. 3 *The structure of TPRV1.* ***(A)*** *TRPV1 chains (colored), Connolly surface of the molecular cavities (grey). The vanilloid pocket is highlighted by the black box. Taken and adapted from Elokely et al., 2015 [335].* ***(B)*** *Linear diagram depicting major structural domains in a TRPV1 subunit. Taken from Liao et al., 2013 [44]. Cap, capsaicin; PI, phosphatidylinositol; RTX, resiniferatoxin*

TRPV1 Activation

As stated above, the activators of the TRPV group are vanilloids. The vanilloid binding site is located between the transmembrane helices. Upon binding, vanilloid initiates a sequence of allosteric modifications, ultimately resulting in the dilation of the selectivity filter and; consequently, the opening of the channel [75]. The most well-known vanilloid activator is capsaicin, which is isolated from chili peppers (*Capsicum sp.*) or cayenne pepper and is responsible for the pungent taste of hot foods [98]. Capsaicin was proposed as an activator in 1997 [70] and remains the most prevalent TRPV1 agonist in use today. Another vanilloid TRPV1 agonist is resiniferatoxin, which occurs naturally in the plant *Euphorbia resinifera* and is also commonly used in laboratory experiments [74],[75]. In addition to these naturally occurring substances, there is a synthetically produced vanilloid antagonist capsazepine that has been developed to inhibit the effects of TRPV1 [99].

TRPV1 is also activated by low pH with a threshold of approximately 6 [77] and by endogenous compounds associated with pain, including lysophosphatidic acid [100] and endocannabinoid anandamide [101],[102]. TRPV1 activation is also influenced by magnesium and barium cations, which have been shown to reduce the action potential in the extracellular region of the channel, thus potentiating its activation [103]. Furthermore, the channel is affected by certain toxins, such as those found in the venom of Chinese bird spiders, which have been shown to induce permanent activation of TRPV1 [104]. TRPV1 is also responsive to high temperatures, with activation occurring above 43 °C [77]. This heat-induced influx of calcium ions into the cell has been shown to elicit a response analogous to that triggered by capsaicin. The precise mechanism underlying this phenomenon has not yet been elucidated; however, a general assumption is made regarding a conformational change within the intracellular region and the outer pore, which is assumed to be independent of vanilloid activation [105].

The influence of TRPV1 on BAT and its activation remains a topic of ongoing debate due to inconsistencies in the literature. There is evidence that TRPV1 activation activates BAT thermogenesis [106] and induces beiging of WAT [107]. On the contrary, some authors have reported that nonthermal activation of TRPV1 leads to a decrease in thermogenesis and BAT activity [108],[109].

The decision to study TRPV1 in the context of thermogenesis and immune response was motivated by the thermosensitive properties of this channel, its association with pain, and the ambiguity of its role in the inflammatory process. Given that the experimental component of this doctoral thesis primarily focused on macrophage populations, the following section will focus on their characteristics and functions.

Macrophages

Macrophages are cells classified among the components of innate immunity. These cells exhibit significant heterogeneity depending on the tissue in which they are located.

Consequently, they acquire specific designations, including Kupffer cells in the liver, osteoclasts associated with bone tissue, and microglia in the nervous system. These professional phagocytes fulfil an important role in pathogen engulfment, antigen presentation, wound healing, tissue homeostasis and individual development [110].

In conventional classifications, macrophages are categorized into two primary phenotypes: classically activated M1 macrophages and alternatively activated M2 macrophages. However, this classification is not universally applicable, as it is a highly dynamic array of different subtypes that can exhibit a wide range of properties, from highly similar to distinctly different. The repolarizations and substitutions that occur between these subtypes are influenced by factors such as the tissue environment, the presence of cytokines, and metabolic activity.

M1 macrophages

Classically activated macrophages, also known as pro-inflammatory M1 macrophages, fulfill a pivotal function in host defense. These cells possess the ability to phagocytose pathogens, participate in the inflammatory reaction, and contribute in the antitumor response. The induction of these cells is primarily driven by an inflammatory cytokine environment, with IFNγ being the key inducer. Activation via pattern recognition receptors (PRR) is also very important [111], which is discussed later. These activated macrophages target pathogens by producing bactericidal reactive oxygen species (ROS) and nitric oxide (NO), which is produced by inducible nitric oxide synthase (iNOS) [111],[112]. They amplify the inflammatory response by producing inflammatory cytokines, including IL-1β, IL-6, IL-12, IL-23, and TNFα. Furthermore, M1 macrophages are known to exhibit a high level of presentation of antigen and costimulatory molecules CD80 and CD86 [113],[114].

M2 macrophages

Alternatively activated macrophages, also known as anti-inflammatory M2 macrophages, play a key role in regulating the inflammatory response, facilitating tissue repair and promoting wound healing. In contrast to the simplified classification of M1 macrophages, the spectrum of M2 macrophages is considerably more intricate, encompassing four distinct subtypes: M2a, M2b, M2c and M2d (Fig. 4).

M2a macrophages, which are considered the true alternatively activated macrophages, are predominantly implicated in wound healing due to their role in the production of profibrotic factors [115]. These cells are primarily stimulated by the presence of the cytokines IL-4 and IL-13. Characteristic markers underlying this phenotype include arginase 1 (Arg-1), macrophage mannose receptor (CD206), CD163, transforming growth factor-β (TGF-β), IL-10, and chemokine C-C motif ligand 17 (CCL17) [116],[117]. In particular, the M2a phenotype has been observed to exhibit a negative regulatory influence on the M1 phenotype [118].

The M2b phenotype is referred to as regulatory macrophages, as it maintains the balance of the pro- and anti-inflammatory environment. These cells are induced by a combination of

immune complexes and PRR agonists, such as Toll-like receptor 4 (TLR4) and interleukin 1 receptor (IL-1R). These cells are known to produce pro-inflammatory cytokines, such as IL-1β, IL-6, and TNFα, though to a lesser extent than M1 macrophages. In addition, they exhibit high levels of anti-inflammatory cytokine IL-10 and low IL-12 production [119]. Furthermore, the expression of CD86, CCL1, and TNFSF14 (also known as the LIGHT molecule) has been observed [111],[120],[121].

Macrophages classified as M2c are also referred to as deactivating macrophages. Induction of these cells is facilitated by a high level of IL-10, which they subsequently produce in significant quantities in conjunction with TGF-β. The resulting anti-inflammatory and profibrotic effect is attributed to the actions of these cytokines [117]. Moreover, these cells have demonstrated high efficacy as phagocytes of apoptotic bodies, a feature attributable to the expression of the Mer receptor tyrosine kinase (MerTK). They also express Arg-1, CD163, CD206, or CCR2 [117],[122].

M2d are more commonly referred to as "tumor-associated macrophages" (TAMs). This particular phenotype is induced by co-stimulation of TLR agonists in conjunction with A2 adenosine receptor (A2R) agonists or IL-6. These cells have been observed to produce significant amounts of the cytokines IL-10, TGF-β, and vascular endothelial growth factor (VEGF), while exhibiting relatively lower levels of the inflammatory cytokines IL-1β, IL-12, and TNFα. This profile has been shown to promote increased angiogenesis and contribute to tumor metastasis [117],[122]. Additionally, these cells express characteristic M2 macrophage molecules, such as Arg-1 [123].

Beyond this more fundamental classification, a multitude of other more specific types of macrophages have been identified, including Mhem, which has been shown to be inducible by blood heme and exhibit atheroprotective properties. Mox macrophages, on the other hand, are induced by oxidized lipids and act as antioxidants due to IL-10 and COX-2 [124]. Finally, sympathetic neuron-associated macrophages (SAMs) are a unique subset of macrophages that reside along sympathetic neurons, where they likely play a crucial role in regulating neurotransmitter concentration, as evidenced by their ability to actively take up norepinephrine [125].

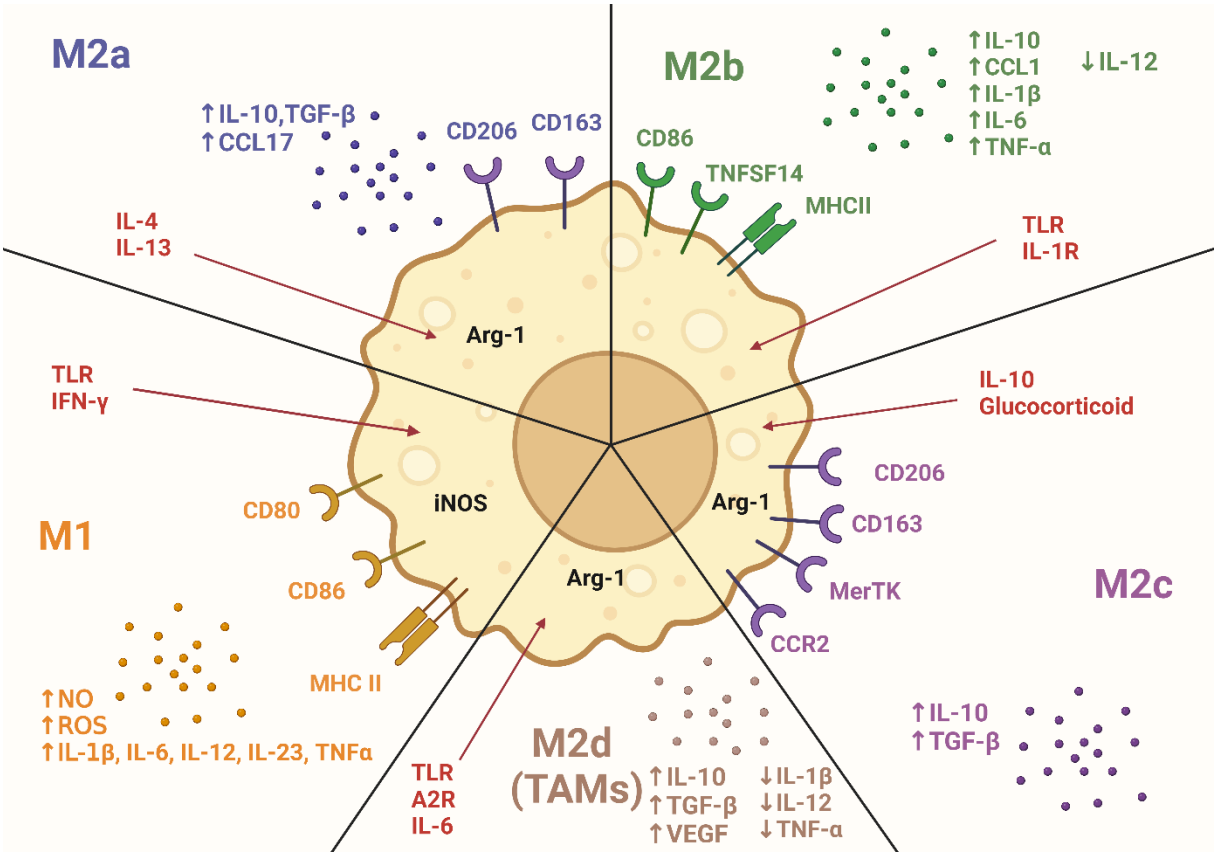


Fig. 4 *Macrophage subtypes (M1 - yellow, M2a - blue, M2b - green, M2c - purple and M2d - brown) and their markers. Red arrows red show the need for stimulation to induce the appropriate phenotype. See text for details. A2R, A2 adenosine receptor; Arg-1, arginase 1; CCL, chemokine C-C motif ligand; CD, cluster of differentiation; IFN-γ, Interferon γ; IL, interleukin; iNOS, Inducible nitric oxide synthase; MerTK, Mer tyrosine kinase; MHCII, major histocompatibility complex class II; NO, nitric oxide; ROS, reactive oxygen species; TGF-β transforming growth factor β; TLR, Toll-like receptor; TNFSF14, tumor necrosis factor superfamily member 14; TNFα, tumor necrosis factor α; VEGF, vascular endothelial growth factor. Created in Biorender.com*

TRPV1 on immune cells

Although TRPV1 is a non-selective channel, permeability to calcium Ca^{2+} cations plays the most significant role in immune cells. These ions function as second messengers, contributing to the activation of Ca^{2+}-dependent kinases and transcription factors [126].

The role of TRPV1 as a nociceptor is well established and has therefore been the focus of many studies in various pathological conditions associated with pain [127],[128] and chronic diseases. In a model of chronic obstructive pulmonary disease, plant flavonoids have been shown to reduce TRPV1 expression, leading to a concomitant decrease in pro-inflammatory cytokines IL-1β, IL-6 and TNFα, as well as NO concentration [129]. Additionally, in a mouse

model of asthma, suppression of TRPV1 expression has been shown to lead to reduced lung inflammation [130].

In immune cells, the role of TRPV1 is best understood in T lymphocytes. TRPV1 activation contributes to an increase in Ca^{2+} concentration after TCR stimulation but also plays a role in downstream TCR signaling [89]. Inhibition or knockout (KO) of TRPV1 results in impaired nuclear factor-kappa-light-chain-enhancer of activated B cells (NF-κB), mitogen-activated protein (MAP) kinase and nuclear factor of activated T cells (NFAT) signaling following TCR activation [88],[131]. This results in reduced production of the cytokines IFNγ, IL-17A and TNFα [88],[89]. Further evidence suggests that after TCR activation, TRPV1 stimulation is responsible for shifting the balance of T helper (Th) 1/Th2 cells towards the anti-inflammatory Th2, through activation of the transcription factor NFATc2 [97]. In summary, evidence suggests that activation of TRPV1 in T cells attenuates the pro-inflammatory response and supports the Th2 response, but there are also conflicting results. Bertin et al. show in their study that TRPV1-expressing CD4+ T cells are active in a model of colonic inflammation and colitis, and that suppression of TRPV1 activity with an antagonist alleviated inflammation in their models [88],[132].

In the context of DCs, the negative effect of TRPV1 activation on their differentiation and maturation has been described. Furthermore, suppressed production of cytokines IL-6 and IL-12 has been documented, while anti-inflammatory cytokines IL-10 and TGF-β are increased [95],[133]. This suggests that in DCs, TRPV1 activation may favor an anti-inflammatory immune response.

In neutrophils, a functional TRPV1 response has been shown to induce Ca^{2+} influx upon activation [91],[134]. Mast cells show higher levels of TRPV1 in mastocytosis compared to controls, suggesting involvement in mast cell-mediated inflammation [93]. However, in the case of granulocytes, the field of research is more uncharted. In the case of B cells or NK cells, the situation is perhaps even more unexplored, so more scientific publications are needed before firm conclusions can be drawn.

TRPV1 on macrophages

There is a significant amount of literature investigating the effect of TRPV1 on the functionality and the phenotype of macrophages. However, the findings from these studies are even more conflicting than those from studies of other immune cell types. Some articles suggest a clear pro-inflammatory role for TRPV1, while others label TRPV1 activity as anti-inflammatory. The expression of TRPV1 in mouse macrophages was first confirmed in 2017 [96], and its presence in human macrophages was acknowledged in 2022 [97].

Initial studies indicated that TRPV1 activity was associated with a pro-inflammatory response. Capsaicin-activated TRPV1 promoted neuroimmune interactions that led to an increase in M1 macrophage polarization [135]. Furthermore, in the same year, another study was published

showing the anti-inflammatory effect of TRPV1 antagonists on lipopolysaccharide (LPS)-induced inflammation in macrophages [96]. However, subsequent studies have revealed a contradictory relationship, suggesting a potential anti-inflammatory role for TRPV1. In the TRPV1 KO model, inflammatory macrophage-mediated kidney injury was exacerbated [136]. Additionally, TRPV1 activation was found to reduce production of IL-6 and TNFα by microglia in a model of Alzheimer's disease [137]. Anti-inflammatory properties have also been demonstrated in microglia obtained from patients with multiple sclerosis. A mutation in the TRPV1 gene that resulted in increased expression and activity of TRPV1 was found to reduce the TNFα production. Moreover, pretreatment of microglia with a TRPV1 agonist led to lower levels of IL-6 and TNFα in LPS-induced inflammation [138]. In a rat model of osteoarthritis, when capsaicin was injected into the joint connection, a lower expression of M1 macrophage markers (CD80, IL-6, iNOS) was observed, as well as in capsaicin-activated TRPV1 macrophages stimulated by LPS, but the expression of M2 markers remained unchanged [139]. Similar results were previously observed by Bok et al. in a rat model of Parkinson's disease. In this study, the authors observed a decrease in pro-inflammatory M1 markers (IL-1β, IL-6, iNOS) in LPS-induced inflammation treated with capsaicin, accompanied by an increase in M2 markers CD206 and Arg-1. The use of the antagonist capsazepine reversed these effects [140].

However, there are more recent articles that support the pro-inflammatory effect of TRPV1. Zhang et al. published a paper that demonstrated the regulation of the NLRP3 inflammasome by the influx of Ca^{2+} cations through TRPV1 [126]. In the case of Chikungunya virus (CHIKV) infection, M1 macrophages are known to be activated, and an increased expression of TRPV1 has been confirmed. The application of the agonist resiniferatoxin has been demonstrated to enhance the production of IL-6 and TNFα, thereby underscoring the pro-inflammatory function of TRPV1 in macrophages [141]. Moreover, inactivation of TRPV1 has been shown to alleviate CHIKV- induced pain in murine models [142].

The literature on TRPV1 role in immune responses is filled with conflicting results, which can be attributed to the use of whole-body KO animals, leading to a loss of specificity in the results on the role of TRPV1 in immune cells, as the loss of TRPV1 primarily affects neurons. Additionally, the close cooperation between nociceptive neurons and the immune system complicates the interpretation of these results [143],[144]. Another important factor is the timing of TRPV1 stimulation relative to the course of the inflammatory response. The existing literature suggests that different results can be obtained based on the timing of TRPV1 activation, depending on whether it occurs before, during, or after the acute inflammatory response. Confirmation of this hypothesis would provide a promising therapeutic approach.

Cold adaptation

Studying cold as an opposite approach to mimicking warm stimuli may seem contradictory. However, both processes share similar underlying mechanisms, although the results are markedly different. The second part of my experimental work focuses on the response of the

immune system to cold acclimatization, and in this context, it is important to introduce some basic knowledge about how the immune system adapts to cold exposure.

The immune system and the cold temperature

During exposure of the organism to low temperatures, alterations occur in all tissues and systems with the primary objective of maintaining constant homeostasis. This includes the immune system, among other body systems, each of which must perform its designated function. Consequently, the impact of cold is a subject of extensive research. The effects of cold vary depending on the duration and intensity of the stimulus. Brief cold exposure can elicit an acute stress reaction, while mild exposure to cold that persists for an extended period can induce a state of tolerance, adaptation, and subsequent acclimatization.

Various models have been employed to investigate the impact of low temperature on the immune system. Cold has been demonstrated to influence the development and subsequent survival of insects, as well as their reproductive ability [145]. In the fruit fly (*Drosophila melanogaster*), cold alone is sufficient to activate the immune system, but only to the extent that it compensates for cold damage and can effectively defend against pathogens [146]. In the context of fish, temperature plays a pivotal role. In tilapia *(Oreochromis aureus*), acute cold stress has been shown to suppress leukocyte phagocytic activity and induce an immunosuppressive state [147]. In pufferfish (*Takifugu obscurus*), low-temperature exposure has been observed to induce oxidative stress, apoptosis, and DNA damage [148]. A decrease in the total number of leukocytes has been observed in common carp (*Cyprinus carpio*) [149] and in orange-spotted grouper (*Epinephelus coioides*) [150]. Cold temperatures have also been shown to exert an immunosuppressive effect on amphibians, making them more susceptible to fungal infection [151]. In summary, it can be posited that ectothermic animals utilize ambient temperature to effectively modulate the immune system, a strategy that is particularly advantageous to them at a given moment. However, it is noteworthy that pathogens and parasites have evolved analogous mechanisms [152].

In endothermic animals, maintaining core body temperature is crucial, which may lead to the assumption that the immune system is not directly impacted. However, this assertion is not entirely accurate. The objective of this study is to elucidate the impact of altered temperatures, stress, and altered neuroimmune pathways on immune function. Ambient temperature does not exert an influence on the prenatal development of the immune system. It is primarily postnatal environmental conditions and physiological responses that shape immune function and adaptation to temperature changes [153]. For instance, birds that do not migrate face the challenge of allocating more resources toward thermogenesis to maintain body temperature or toward immune system functions to protect against infections. Exposure to cold has been demonstrated to disrupt metabolic balance and slow the natural immune response of these animals. This trade-off can have significant implications for their survival and overall health, as they must balance energy expenditure for thermoregulation with the need to mount an effective immune defense against pathogens [154]. In general, cold

stress has been shown to have a negative effect on the immune system. In rats, short-term exposure to low temperatures has been documented to induce suppression of innate immunity [155],[156], dysregulation of adaptive immunity, and decrease in regulatory T cells (Treg) [156]. Belay et al. concluded that cold stress increases susceptibility to infection and that the immune system responds by inducing a pro-inflammatory response. This response is characterized by an increase in the production of NO, IL-1β, IL-6, and TNFα by macrophages [157]. Similarly, Sánchez-Gloria et al. also observed increased levels of inflammatory cytokines after exposure to cold in the pulmonary arterial hypertension model [158]. An increased susceptibility to infection has also been described in humans, where exposure to cold alters nasal antiviral immunity [159].

Long-term cold exposure; however, tends to induce anti-inflammatory immune phenotypes, such as M2 macrophages and Th2 immune response [160]. The M2 macrophage phenotype is facilitated by IL-4 produced by eosinophils and innate lymphoid cells 2 (ILC2) [161]. Macrophages then exert regulatory functions on spleen cells [162] and stimulate Th cells to produce regulatory cytokines [163]. This results in a decrease in the production of pro-inflammatory cytokines, such as IL-1β and IL-17, accompanied by the induction of IL-10 [164]. Mild cold acclimatization has been shown to mitigate the detrimental effects of acute cold stress, as seen in broilers, where it attenuated intestinal inflammation and immune dysfunction [165],[166]. The protective effect of cold acclimatization has also been described in the context of cardiac muscle in a model of myocardial infarction [167]. Furthermore, cold exposure has been shown to drive immunological reprogramming that protects against central nervous system autoimmunity [168]. Additionally, cold-activated BAT has been shown to facilitate glucose uptake, thereby slowing tumor growth [169]. These findings support the hypothesis that acute cold stress exerts a detrimental effect, while mild cold adaptation confers a beneficial effect.

The scientific literature contains only a limited number of studies on the effect of cold exposure on the immune system in humans. However, a substantial body of research has been dedicated to comparing various physiological aspects, hormone levels, and metabolic functions. The human physiological response to cold exposure has been comprehensively delineated [170]. Notably, these studies are predominantly conducted in athletes, where the relationship between physical activity and cold adaptation has been a research subject [171],[172]. Previous studies have suggested that prolonged exposure to low temperatures may result in an increase in the number of B and T cells [173]. It is now well established that exposure to cold induces human Tregs [174]. Furthermore, regular exposure to cold showers has been shown to enhance both humoral and cell-mediated immunity, increasing levels of immunoglobulins and cytokines IL-2 and IL-4 [175]. A study by Dugué and Leppänen revealed that habitual winter swimmers exhibited a decrease in LPS-induced release of IL-1β and IL-6 [176]. However, it should be noted that these cytokine levels remained unaltered in the absence of stimulation [177]. The collective findings of these studies suggest a beneficial effect of cold adaptation on the human body and its immune system. However, it is important to

note that extreme physical activity in demanding thermal conditions can be detrimental to health [178]. However, research on the effects of regular cold immersion has yielded equivocal results [179]–[181]. This variability can be attributed to substantial differences in participant demographics and study methodologies.

Immune cells involved in cold adaptation

In this chapter, the focus will be on the individual types of immune cells and their influence on the beiging of adipose tissue, BAT activation, and thermogenesis itself. The induction of beiging and the thermogenic effect of BAT are influenced by various types of immune cells, including macrophages, eosinophils, ILC2, mast cells, Tregs, and invariant natural killer T (iNKT) cells. The fundamental influence of gamma delta T (γδ T) cells has also been described. Type 2 cytokines have been identified to be of particular importance; however, the role of cytokines IL-6, IL-17 and TNFα is also significant.

Activated iNKTs in adipose tissue have been shown to maintain an anti-inflammatory environment by producing IL-4, IL-10, and IL-13, thereby inhibiting pro-inflammatory cell infiltration. The influence of iNKTs extends to Treg cells, with IL-2 production playing a pivotal role in this process [182]. Furthermore, these cells contribute to the beiging and activation of adipose tissue through the regulation of fibroblast growth factor 21 (FGF-21) [183]. This factor is also produced in adipose tissue by thermogenic adipocytes and is regulated by noradrenergic signaling and thermogenic gene expression [184]. Moreover, FGF-21, produced by adipocytes, has been reported to increase the expression of CCL11, which is a significant eosinophil chemoattractant [160]. The attraction of eosinophils is also ensured by ILC2 by producing IL-5, which responds to IL-33 produced by stromal cells [185]. Activated ILCs2 produces IL-5, which is essential for eosinophils. Furthermore, ILCs2 secrete the methionine-enkephalin peptide, which acts directly on adipocytes and induces UCP1 expression [186]. In turn, eosinophils produce IL-5, IL-13 and mainly IL-4, which seems to be the most important cytokine during thermoregulation. It is not only used in the induction and maintenance of M2 macrophages, but it also participates in the proliferation and differentiation of adipocyte precursors to the beige thermogenic type [161],[187]. Treg cells also influence adipose tissue thermogenesis by supporting the M2 macrophage phenotype and the expression of thermogenic genes in adipocytes [188]. The expansion of these cells within adipose tissue depends on IL-33, which binds to the ST2 receptor [189]. The negative regulatory function is performed by mast cells, which secrete serotonin, thus impeding the differentiation of adipocyte precursors into beige thermogenic cells [190].

Macrophages

Nguyen et al. initially postulated that macrophages in adipose tissue, under the influence of IL-4 in cold conditions, directly produce NE and thus induce thermogenesis in adipocytes through adrenergic signaling [191]. However, a subsequent study by Fischer et al. provided evidence that IL-4 does not stimulate resident macrophages in adipose tissue, thus

invalidating the initial assumption that M2 macrophages influence thermogenesis through this mechanism [192]. A subsequent study by Pirzgalska et al. revealed the discovery of SAMs. Although these macrophages cannot produce NE, they have been shown to be able to take it and degrade it. This finding suggests that the SAM subtype of macrophages plays a regulatory role in NE levels, thereby affecting thermogenic activities [125]. Another neuroimmune mechanism involves the ability of macrophages to support sympathetic innervation of BAT, thus increasing its thermogenic capacity [193].

A multitude of additional studies have indicated the significance of macrophages in non-shivering thermogenesis. One of such mechanisms involves the production of PPARγ ligands [194], a crucial factor in UCP1 expression and the differentiation of brown and beige adipocytes [27]. Another molecule produced by macrophages that regulates the expression of thermogenesis genes is the chemokine C-X-C motif ligand (CXCL) 5. Remarkably, this chemokine is produced in adipose tissue by M1 macrophages after β-adrenergic signaling, thus inducing the expression of UCP1 in adipocytes [195]. In M2 macrophages, the detection of another molecule that activates thermogenesis was made, the macrophage cytokine Slit3. This molecule is a key factor in the macrophage–Slit3–sympathetic neuron–adipocyte signaling axis, functioning as a regulator of long-term cold adaptation [196]. Additionally, M2 macrophages exert a neuroindependent influence on thermogenesis by suppressing the expression of the transcription factor Ets1 in adipocytes, which facilitates the beiging process [197]. However, it should be noted that the extent of macrophage involvement in adipose tissue remodeling remains debated. Boulet et al. argue that the direct involvement of macrophages is not essential for the formation of thermogenic adipocytes. However, they admit that during prolonged cold exposure, the M2 population is enriched in BAT, which ensures better remodeling and significant homeostasis [198]. Another important function of macrophages in BAT is their ability to remove extracellular vesicles containing oxidatively damaged mitochondrial components, thereby preventing disruptions in the thermogenic program [199]. The remodeling of human adipose tissue by macrophages after cold exposure was further documented by Finlin et al. In this study; however, the authors did not identify macrophages as direct initiators of thermogenesis. It is noteworthy that in this publication, the authors observed the presence of UCP1 in CD163-positive macrophages [200].

Gamma delta T cells

γδ T cells are a small population of invariant T lymphocytes that express the γδ T cell receptor (TCR). Although they are in a minority in the body (less than 5% of T cells in peripheral blood), their role in defense is considerable. Their frequency increases especially in peripheral tissues, where it reaches up to 50%. Notably, these cells are capable of rapid immune responses, recognizing antigens presented by non-classical MHC molecules, lipid antigens presented by CD1d, or various phosphoantigens or pyrophosphate molecules presented by butyrophilin or butyrophilin-like proteins. Additionally, these cells also express TLRs and NK cell activating receptors. Their response resembles that of innate immunity rather than that of adaptive lymphocytes [201],[202].

Two distinct mechanisms have been elucidated through which these cells contribute to thermogenesis in adipose tissue. The initial mechanism, described by Kohlgruber et al., is based on the production of IL-17A and TNFα by γδ T cells. These cytokines induce the production of IL-33 by stromal cells, which in turn promotes the proliferation of Treg cells and activates ILC2. Furthermore, it has been observed that these cytokines exert a direct effect on adipocytes during periods of exposure to cold, leading to increased expression of thermogenic genes and those involved in lipid breakdown [189].

The second mechanism described by Hu et al. is the production of IL-17F, another isoform of IL-17. This cytokine exerts its influence on stromal cells triggering the production of TGF-β. The resulting increase in TGF-β levels enhances sympathetic innervation of BAT. Subsequent release of NE from sympathetic nerves induces non-shivering thermogenesis [203]. Additionally, the direct role of TGF-β receptors in regulating beiging of WAT has been well documented [204].

However, an alternative set of results was obtained by a different research group. Teijeiro et al. demonstrated that inhibition of IL-17A signaling also induces thermogenesis. Furthermore, deletion of the IL-17RA receptor has been shown to increase UCP1 expression in adipose tissue [205]. A potential explanation for these discrepancies may reside in the temperature conditions under which the experimental animals are maintained and the extent of cold exposure that stimulates non-shivering thermogenesis. In the Teijeiro model, control animals were maintained at a room temperature of 20-22 °C, which is comparable to the temperature at which mice experience cold stress. In contrast, Hu et al. and Kohlgruber et al. kept control mice at a thermoneutral temperature of 30 °C. Another potential explanation is related to the mechanism of signaling via IL-17 receptors, which are known to exhibit significant variability [206]. Despite these conflicting results, the role of γδ T cells in adipose tissue and thermogenesis is widely accepted. However, these disparate results underscore the need for further research and elucidation of the mechanisms involved in the function of γδ T cells in thermogenesis.

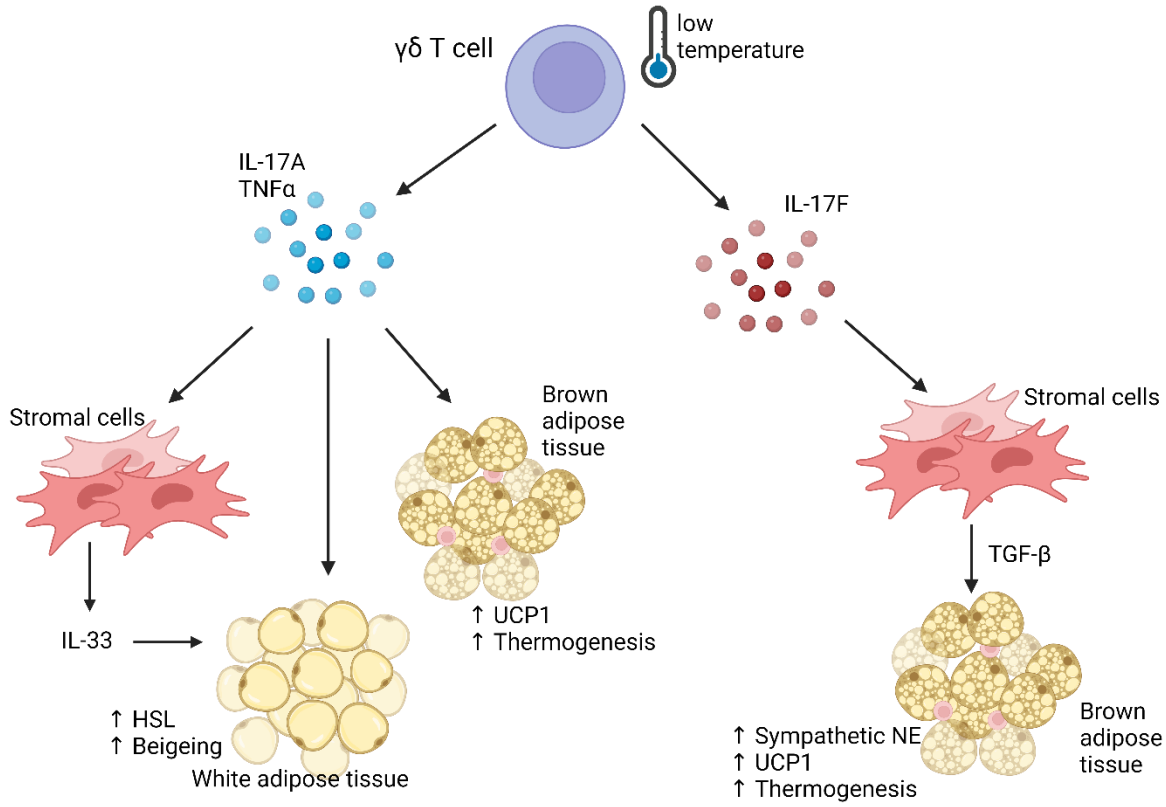


Fig. 5 *Functional properties of γδ T cells after cold exposure. γδ T cells produce the cytokines IL-17A and TNFα, which affect stromal cells, white and brown adipose tissue. In stromal cells, they induce the expression of IL-33, which further promotes the infiltration of adipose tissue by immune cells and promotes the beiging of adipose tissue. In white and brown adipose tissue, cytokines IL-17A and TNFα increase hormone-sensitive lipase activity, induce beiging and UCP1 expression, and increase thermogenesis. Production of IL-17F by γδ T cells affects stromal cells, which then produce TGF-β. This growth factor helps the sympathetic innervation of adipose tissue and the subsequent production of norepinephrine. This increases UCP1 expression and overall thermogenesis. HSL, hormone-sensitive lipase; IL, interleukin; TGF-β, transforming growth factor β; TNFα, tumor necrosis factor α; UCP1, uncoupling protein 1. Created in Biorender.com*

Inflammation

The study of thermogenic stimuli is crucial for understanding the mechanisms of temperature changes and their influence on the immune system. However, the results obtained during the induction of a "danger" or infection are indispensable for elucidating the functionality of the immune system in these states. In the experimental section of this study, the induction of inflammation by TLR ligands was utilized to activate the immune system. In this final chapter of the introduction, the topic of inflammation and its triggering by TLRs will be addressed.

A precise and universally accepted definition of inflammation remains imprecise and debated. Inflammation can be conceptualized as a series of defensive responses that, while protective, may also cause harm to the individual. It is a comprehensive mechanism encompassing natural immune processes that predominantly respond to tissue damage caused by pathogenic agents. These agents encompass a wide spectrum of biological entities, including viruses, bacteria, yeasts, and fungi, as well as eukaryotic protozoa and multicellular parasites. However, inflammation also plays a role in antitumor immunity and autoimmune diseases. Additionally, there is a distinct category of sterile inflammation, a form of pathogen-free inflammation triggered by mechanical trauma, ischemia, stress, or environmental factors such as ultraviolet radiation [207],[208]. The initiation of inflammatory pathways requires their activation, which is typically accompanied by recognition of danger.

Pattern recognition receptors (PRRs)

PRRs are responsible for danger recognition. These receptors are predominantly present in cells of innate immunity; however, they are also found in cells of adaptive immunity, epithelial cells, endothelium, fibroblasts, and astrocytes [209],[210]. These receptors are capable of recognizing two distinct classes of molecules. The first class of molecules are pathogen-associated molecular patterns (PAMPs), which are associated with microbial pathogens. The second class consists of alarmins, which are more commonly referred to as damage/danger-associated molecular patterns (DAMPs). DAMPs are associated with host cell molecules that are released upon damage or during cell death. According to their localization, PRRs are classified into two distinct categories: membrane-bound and cytoplasmic. Cytoplasmic PRRs include NOD-like receptors (NLR) and RIG-I-like receptors (RLR). The most well-known and least well-studied NLRs are NOD1 and NOD2 receptors, which recognize components of bacteria [209],[210] RLRs primarily function in antiviral signaling, and their activation results in the release of pro-inflammatory cytokines and type I IFN [210]. Membrane-bound PRRs encompass TLRs and C-type lectin receptors (CLRs). CLRs are predominantly utilized in fungal recognition; however, other PAMPs capable of their activation have also been identified. The most prominent among these are the mannose receptor and Dectin 1 and 2 [209],[210]. TLRs represent the most extensively studied group of PRRs, and since their stimulation was the tool for inducing inflammation in the experimental part of the dissertation, their more thorough examination is reserved for the following chapter.

Toll-like receptors (TLRs)

TLRs were discovered as homologues of Toll receptors for innate immunity in drosophila. These receptors contain a conserved extracellular domain characterized by a high density of leucine-rich repeats, which facilitates the recognition of distinct PAMPs and DAMPs [211]. To date, 10 human and 12 mouse TLRs have been identified. These TLRs have been found to occur both in the cell membrane and in intracellular vesicles, depending on the specific type of TLR in question. These receptors form homodimers or heterodimers, which is a critical step in the recruitment of adapter proteins and subsequent signaling pathways. Two signaling pathways

have been identified: the myeloid differentiation primary response 88 (MyD88)-dependent pathway and the TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathway. The MyD88-dependent pathway is used by all types of TLRs except TLR3. The TRIF-dependent pathway is utilized by TLR3, though it can also be employed as signaling through TLR4. The process is further facilitated by the involvement of additional adapter proteins and kinases, ultimately culminating in the binding of the transcription factor NF-κB and its subsequent translocation to the nucleus. In the nucleus, NF-κB induces the expression of pro-inflammatory cytokines. In the case of viruses recognizing TLRs, the transcription factors interferon regulatory factor (IRF) 3 and 7 are activated, causing the transcription of type I IFN [209],[210],[212]. Individual receptors, along with their localization, ligands, and adapter pathways, are illustrated in Figure 6B. The signaling pathways are simplified in Figure 6A. The subsequent introduction will focus on TLR2 and TLR4, which were utilized to induce inflammation in the experimental section.

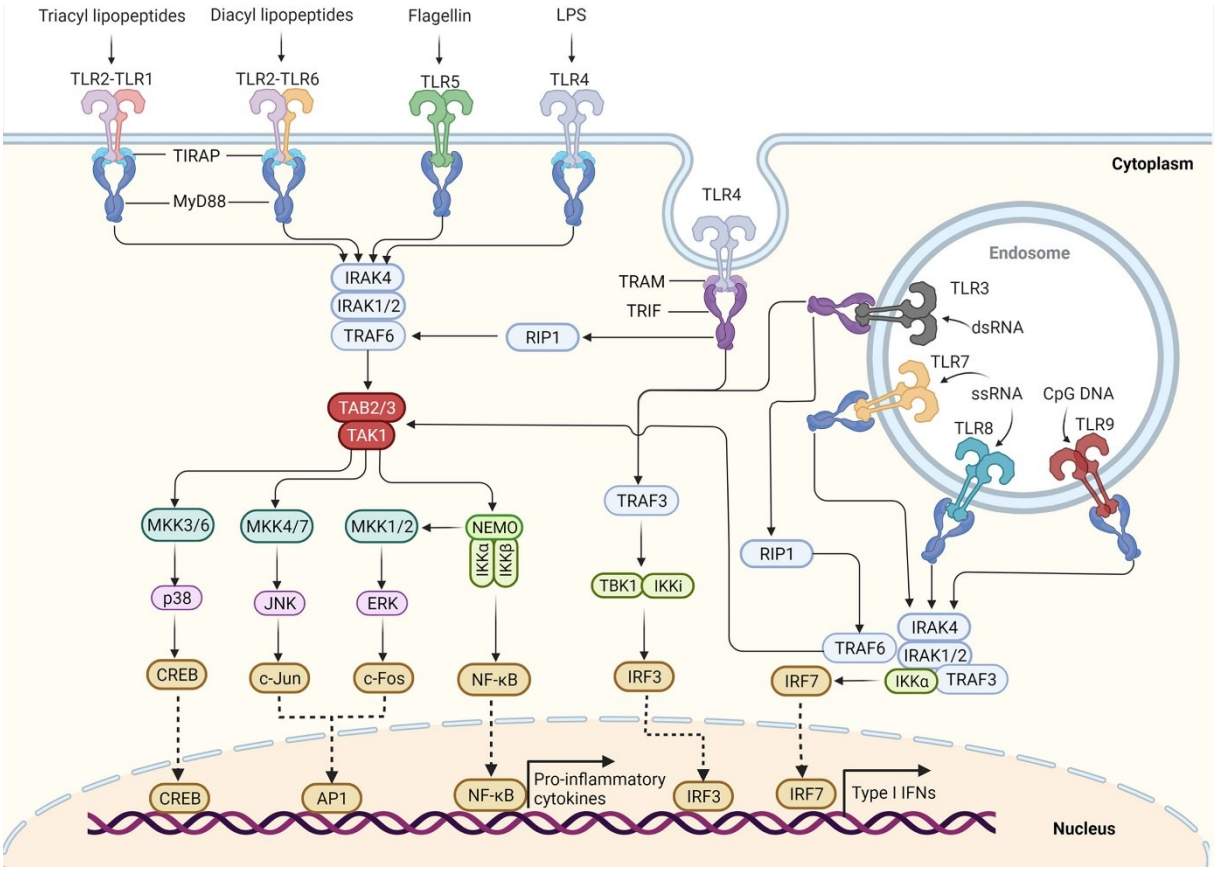
Toll-like receptor 2 (TLR2)

TLR2 is expressed on the cytoplasmic membrane of nearly all types of immune cells, including monocytes, macrophages, DCs, granulocytes, NK cells, B and T cells, as well as Treg and γδ T cells. It has been observed to recognize a variety of molecules, including glycolipids and teichoic acid of Gram-positive bacteria, yeast zymosan, and lipoproteins of Mycoplasma. Notably, TLR2 frequently forms heterodimers with TLR1 and TLR6. Upon recognition of PAMPs, innate immune cells trigger phagocytosis and the production of pro-inflammatory cytokines, including TNFα, IL-6, IL-8, and IL-12. Synthetic lipopeptides, such as Pam2CSK4, are frequently utilized to stimulate these processes in laboratory settings [209],[210],[212],[213].

Toll-like receptor 4 (TLR4)

This receptor is the most extensively studied and described of the TLRs. It is predominantly located on the cytoplasmic membrane; however, it can undergo internalization, thereby participating in signaling that results in the production of type I interferons. The most well-known natural ligand of TLR4 is LPS, a significant component of the gram-negative bacteria, whose binding is facilitated by the CD14 molecule. However, it is important to note that TLR4 can also be activated by a number of DAMPs. Its expression is predominantly observed in myeloid cells, such as monocytes, macrophages, and DCs, although it is also present in B cells and certain T lymphocytes. Additionally, TLR4 can be found in epithelial and endothelial cells. Its expression has been confirmed in many types of tissues, including muscle, brain, liver, lung, and adipose tissue. Activation can be conducted through both the MyD88-dependent pathway and after receptor internalization through the TRIF-dependent pathway. In both cases, relevant transcription factors are activated, which, in the case of the MyD88-dependent pathway, are NF-κB and AP-1. Subsequently, these transcription factors induce the expression of pro-inflammatory cytokines, such as TNFα and IL-6, as well as type III IFN. In the case of the TRIF-dependent pathway, type I IFN and chemokines that activate innate immunity and Th1 adaptive immunity are produced [209],[210],[212],[214].

A



B

Toll-like receptor	TLR localization	Adapter protein	Ligands	Origin of ligands
TLR1	Plasma membrane	MyD88	Lipopeptide	Bacteria
TLR2	Plasma membrane	MyD88	Lipopeptide, Teichoic acid, Glycolipids, Zymosan	Bacteria, Fungi, *Trypanosoma cruzi, Neisseria*
TLR3	Endosome	TRIF	dsRNA, poly I:C	Viruses
TLR4	Plasma membrane Endosome	MyD88 TRIF	LPS, Hyaluronic acid	Bacteria
TLR5	Plasma membrane	MyD88	Flagellin	Bacteria
TLR6	Plasma membrane	MyD88	Lipopeptide, Zymosan	*Mycoplasma*
TLR7	Endosome	MyD88	ssRNA	Viruses
TLR8	Endosome	MyD88	ssRNA	Viruses
TLR9	Endosome	MyD88	CpG DNA	Bacteria, Viruses, *Malaria*
TLR10	Plasma membrane	MyD88	Lipopeptide	*Borrelia burgdorferi, Listeria monocytogenes*
TLR11	Endosome	MyD88	Profilin-like protein	*Toxoplasma gondii*
TLR12	Endosome	MyD88	Profilin-like protein	*Toxoplasma gondii*
TLR13	Endosome	MyD88	23S rRNA	Bacteria

Fig. 6 (A) *Localization and signaling pathways of Toll-like receptors (TLR). TLR1-TLR2 and TLR4-TLR6 are found mainly on the cytoplasmic membrane, where they recognize membrane components of pathogens, while TLR3 and TLR7-9 are localized in endosomes, where they bind nucleic acids. TLR4 can also be endocytosed into endosomes. After binding of the respective ligands to TLRs, TLRs signaling is initiated by receptor dimerization, which leads to the involvement of the adapter proteins MyD88 (in the case of TLR1-TLR2 and TLR4-9) or TRIF (TLR3-4). Subsequent MyD88-dependent signaling pathways lead to the activation of the transcription factors CREB, AP1, and mainly NF-κB, which induces the expression of pro-inflammatory cytokines. The TRIF-dependent pathway activates the transcription factories IRF3 and IRF7, leading to the induction of Type I IFN. Other proteins involved in signaling pathways are also shown in the diagram. Taken from Duan et al. 2022* ***[209]*** ***(B)*** *Table of TLRs, their cellular location, adaptor protein and major ligands with their source. Taken and adapted from Vijay 2018* ***[212]***

TLR2 and TLR4 in temperature regulation

The expression of both TLR2 and TLR4 has been documented in adipose tissue. It has been observed that stimulation of these TLRs can exert an opposite effect on adipogenic differentiation [215]. Moreover, TLR4 activation has been proposed as a potential disruptor of adaptive thermogenesis [216]. Furthermore, mice deficient in TLR2 or TLR4 have demonstrated altered thermogenesis [217]. It was reported that the activation of TLR4, TLR2, and NOD1 leads in BAT to decreased UCP1 expression and decreased mitochondrial biogenesis [218],[219]. On the contrary, inhibition of TLR4 signaling has been shown to promote beiging of adipose tissue [220]. Furthermore, activation of TLR4 in eosinophils in adipose tissue has been shown to negatively affect their production of Th2 cytokines and the promotion the M2 phenotype of macrophages [221]. Collectively, these observations indicate that inflammation induced through TLRs may exert a regulatory influence on thermoregulatory activities.

In addition, previous chapters have delineated the impact of cold exposure on immune and inflammatory responses. It is also noteworthy that cold has been observed to affect platelets during storage, leading to a decrease in TLR2 and TLR4 expression [222]. In the context of the airways, exposure to cold air has been demonstrated to influence TLR4 activation, resulting in enhanced mucin secretion [223]. Furthermore, cold exposure associated with TRPV1 capsaicin-activation, increased TLR2 and TLR4 expression in a rat model of colorectal cancer [224].

The interplay between TLR4 and TRPV1 has been identified as a contributing factor to neuropathic pain [225]. The hypothesis that LPS-stimulated TLR4 modulates TRPV1 channel sensitization has been proposed [226]. Neuronal research suggests that there is an interaction between TLR4 and TRPV1 that leads to modulation of LPS-induced inflammation by TRPV1 [227] and vice versa [228]. This is particularly relevant in light of the contradictory results observed in studies examining the role of TRPV1 in LPS-induced inflammation in macrophages

[96],[126],[137],[138]. Unraveling the role of this temperature sensor appears to be of paramount importance.

By elucidating mechanism of regulation, it may be possible to modulate the TRPV1 channel, thereby affecting TLR-induced inflammation and neuropathic pain. Similarly, the study of cold exposure and its effect on the immune system, either as a mediator of thermogenesis induction or as an altered immune response to TLR-induced inflammation, is extremely important. These are intricate mechanisms involving interactions between the nervous and immune systems, the understanding of which may lead to applications in clinical medicine. During my doctoral studies, I concentrated on these mechanisms and endeavored to contribute to their comprehension.

Aims

The objective of this study is to broaden the general understanding of the impact of thermogenic stimuli on the immune system and its responses. As primary research, this study provides insights that may guide future clinical investigations. In order to address the interplay between temperature regulation and immune function, the present study focuses on two key aspects. Firstly, the role of the high-temperature receptor TRPV1 in immune responses is examined. Secondly, the impact of cold conditions on immune cell adaptation and thermogenic-immune interactions is investigated.

1) To elucidate the impact of activation of the high-temperature-dependent TRPV1 channel in macrophages

The objective of this study is to investigate the potential implications of TRPV1 activation in macrophages, with a focus on its role in the regulation of immune responses and the associated macrophage phenotype (M1/M2). This work is based on the presence of conflicting data in the literature on this topic. To induce an immune response, an inflammatory environment will be created using the TLR4 ligand LPS. The specific objectives of the study were as follows:

1a) To explore the expression of TRPV1 channel in macrophages and its capsaicin-activated signaling pathways

1b) To determine the phenotype of macrophages after TRPV1 stimulation at various time points

1c) To test TRPV1 stimulation in LPS-activated macrophages with the aim of determining the conditions that promote phenotypic shifts.

2) To elucidate the alterations in the immune system in response to cold exposure

In this study, we employed a long-term rat model of mild cold acclimatization to comprehensively examine changes in the immune system at multiple time points, including both short-term and sustained responses. The primary aims of this study were two: first, to expand the existing body of knowledge about the immune mechanisms that contribute to the physiological response to cold exposure and second, to provide a comprehensive

description of changes in the immune system at different time points after acclimatization to low temperature. The following specific objectives were thus defined:

2a) To describe immune changes at the systemic and local level during cold acclimatization

2b) To uncover key immune regulators involved in the induction of thermogenesis

2c) To verify the data obtained from the rat model in human volunteers

2d) To assess the impact of TLR2-induced inflammation on the cold acclimatization process in adapting animals.

Results

The thesis is based on the following publications:

I. **Vasek D**, Fikarova N, Markova VN, Honc O, Pacakova L, Porubska B, Somova V, Novotny J, Melkes B, Krulova M. **Lipopolysaccharide pretreatment increases the sensitivity of the TRPV1 channel and promotes an anti-inflammatory phenotype of capsaicin-activated macrophages**. J Inflamm (Lond). 2024 May 24;21(1):17. doi: 10.1186/s12950-024-00391-0. PMID: 38790047. (IF=5.1)

II. **Vasek D**, Holicek P, Galatik F, Kratochvilova A, Porubska B, Somova V, Fikarova N, Hajkova M, Prevorovsky M, Zurmanova JM, Krulova M. **Immune response to cold exposure: Role of γδ T cells and TLR2-mediated inflammation**. Eur J Immunol. 2024 Jul 10:e2350897. doi: 10.1002/eji.202350897. PMID: 38988146. (IF=4.5)

Other impacted publications (not included):

- Porubska B, **Vasek D**, Somova V, Hajkova M, Hlaviznova M, Tlapakova T, Holan V, Krulova M. **Sertoli Cells Possess Immunomodulatory Properties and the Ability of Mitochondrial Transfer Similar to Mesenchymal Stromal Cells**. Stem Cell Rev Rep. 2021 Oct;17(5):1905-1916. doi: 10.1007/s12015-021-10197-9. Epub 2021 Jun 11. PMID: 34115315. (IF=4.8)

- Vegrichtova M, Hajkova M, Porubska B, **Vasek D**, Krylov V, Tlapakova T, Krulova M. **Xenogeneic Sertoli cells modulate immune response in an evolutionary distant mouse model through the production of interleukin-10 and PD-1 ligands expression**. Xenotransplantation. 2022 May;29(3):e12742. doi: 10.1111/xen.12742. Epub 2022 Mar 16. PMID: 35297099. (IF=3.9)

- Porubska B, Plevakova M, Fikarova N, **Vasek D**, Somova V, Sanovec O, Simonik O, Komrskova K, Krylov V, Tlapakova T, Krulova M, Krulova M. **Therapeutic potential of Sertoli cells in vivo: alleviation of acute inflammation and improvement of sperm quality.** Stem Cell Res Ther. 2024 Sep 4;15(1):282. doi: 10.1186/s13287-024-03897-9. PMID: 39227878. (IF=7.9)

I confirm that Daniel Vašek, the author of this thesis, has contributed significantly to the publications listed above. Daniel Vašek conducted most of the experimental work and significantly contributed to the manuscript preparation in the case of his first-author publication.

doc. RNDr. Magdaléna Krulová, Ph.D.

I. Lipopolysaccharide pretreatment increases the sensitivity of the TRPV1 channel and promotes an anti-inflammatory phenotype of capsaicin-activated macrophages

A review of the extant scientific literature reveals a certain degree of inconsistency in the results of studies that have investigated the role of TPRV1 in macrophages. The effect of TRPV1 activation on the inflammatory response suggests both pro- and anti-inflammatory effects. In order to elucidate the immunomodulatory mechanisms, we investigated how the TRPV1 agonist capsaicin influences the pro-inflammatory macrophage phenotypes induced by LPS. We also aimed to address the temporal sequence of stimulations based on previously published studies and their conflicting conclusions. We determined changes in surface and intracellular molecules, cytokine production, and signaling cascades leading to the macrophage phenotype M1 or M2. To this end, we employed two distinct sources: the macrophage cell line J774 and bone marrow-derived macrophages. Both cell types were treated with capsaicin before, simultaneously, and after the inflammatory response induced by LPS, a TLR4 ligand. The functional capacity of macrophages was also assessed by infecting stimulated macrophages with the intracellular parasite *Leishmania mexicana*. The results of this study demonstrate that TRPV1 activation elicits distinct macrophage responses influenced by the inflammatory context. LPS pretreatment followed by capsaicin activation prompted increased calcium influx, accompanied by a shift toward an anti-inflammatory M2b-like polarization state. These findings underscore the context-dependent immunomodulatory role of TRPV1 in macrophages by capsaicin in an inflammatory setting and offer novel insights into its potential as a therapeutic target for the treatment of inflammatory diseases by promoting an anti-inflammatory response.

D. Vašek´s contribution on this publication:

I participated in the design of the experiments, their execution, and the subsequent evaluation and visualization of the data. I performed most of the experiments to determine the phenotype of the J774 macrophage line, including their functional assays. I made a substantial contribution to the preparation of the manuscript. The present publication was created with the support of the "Grant Schemes at CU" project (reg. no. CZ.02.2.69/0.0/0.0/19_073/0016935), of which I was the principal investigator. My overall contribution was approximately 45 %.

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Lipopolysaccharide pretreatment increases the sensitivity of the TRPV1 channel and promotes an anti-inflammatory phenotype of capsaicin-activated macrophages

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Abstract

Background The transient receptor potential vanilloid 1 (TRPV1) is well-established in neuronal function, yet its role in immune reactions remains enigmatic. The conflicting data on its inflammatory role, suggesting both pro-inflammatory and anti-inflammatory effects upon TRPV1 stimulation in immune cells, adds complexity. To unravel TRPV1 immunomodulatory mechanisms, we investigated how the TRPV1 agonist capsaicin influences lipopolysaccharide (LPS)-induced pro-inflammatory macrophage phenotypes.

Results Changes in the surface molecules, cytokine production, and signaling cascades linked to the phenotype of M1 or M2 macrophages of the J774 macrophage cell line and bone marrow-derived macrophages, treated with capsaicin before or after the LPS-induced inflammatory reaction were determined. The functional capacity of macrophages was also assessed by infecting the stimulated macrophages with the intracellular parasite *Leishmania mexicana*.

Conclusion Our findings reveal that TRPV1 activation yields distinct macrophage responses influenced by the inflammatory context. LPS pre-treatment followed by capsaicin activation prompted increased calcium influx, accompanied by a shift toward an anti-inflammatory M2b-like polarization state.

Keywords TRPV1, Macrophages, Capsaicin, Inflammation

†Daniel Vašek and Natálie Fikarová contributed equally to this work.

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Introduction

The transient receptor potential vanilloid 1 (TRPV1) is a non-selective cation channel associated with pain signaling and neurogenic inflammation [1, 2]. Nevertheless, accumulating evidence suggests that TRPV1 is also involved in many other processes such as insulin sensitivity, airway hypersensitivity, urinary bladder functions, and notably, regulation of the immune response [3–7]. Activation pathways of nociceptive TRPV channels in neurons have been widely studied [8–10], but their exact role in immune cells remains largely unknown, and moreover published results are often contradictory.

Various endogenous and exogenous molecules interacting with the TRPV1 have been identified. Namely inflammatory molecules [9], cytokines, hormones [5, 11], opioids [12], growth factors, and miRNAs [13], along with mechanical stimuli, UV radiation, decreased pH, and increased temperature [14]. Among numerous exogenous modulators, resiniferatoxin, capsaicin or its antagonist capsazepine are well established [15]. The broad spectrum of TRPV1 modulating molecules indicates a complex regulation of its action, and, indeed, TRPV1 has been demonstrated to be an active player in the pleiotropic immune network. Regarding the role of TRPV1 in the immune system, its activation was initially associated with the induction of an inflammatory response [16]. However, later studies showed that TRPV1 stimulation at the site of ongoing inflammation suppressed the pro-inflammatory effect or even led to the production of anti-inflammatory cytokines. In addition, TRPV1 antagonists were able to suppress the release of inflammatory molecules in a LPS-mediated inflammation in murine macrophages [17] and TRPV1 knockout mice showed an aggravated inflammatory response after LPS injection [18].

The expression of TRPV1 has been confirmed in various immune cells, including T lymphocytes, macrophages, natural killer cells, dendritic cells, and neutrophils [10]. Unfortunately, the role of TRPV1 activation in individual immune populations remains controversial. For example, activated TRPV1 channel regulates the level of intracellular calcium in T lymphocytes, playing a key role in TCR signaling, and possibly participate in T cell development in the thymus [19, 20]. On the contrary, oral administration of capsaicin suppressed the activation of autoreactive T cells in the pancreatic lymph nodes and protected mice from the development of type 1 diabetes. This suppression was specifically mediated through macrophages [21]. Furthermore, the contribution of TRP channels to the macrophage polarization into the alternative M2 phenotype has been documented [22]. An important role for antigen-presenting cells was shown in mice with TRPV1 gain-of-function mutation. Duo et al. (2020) demonstrated that a constitutively active TRPV1 channel exacerbated DSS-induced colitis, and consequently, patients with IBD showed significantly enhanced expression of TRPV1 protein in infiltrating immune cells in the lamina propria of the inflamed colon [23]. Yet another important role of TRPV1 function in cancer growth and metastasis has also been documented; it can promote or suppress cancer cell death, depending on the type and environment [24]. The crucial role of macrophages in cancerogenesis has been shown in a mouse model of colorectal carcinoma, where gain of function of TRPV1 increased tumor incidence and burden. The effect of TRPV1 overexpression on the M1/ M2 pathways was confirmed, creating a deleterious microenvironment for tumorigenesis [25].

Together, the current research confirmed TRPV1 as an important physiological and pathophysiological molecule that significantly regulates macrophage function. To clarify the role of TRPV1 in the inflammatory response, in the present study, macrophages were stimulated with capsaicin simultaneously, before or after the LPS-induced inflammatory response and signaling cascades and various markers and functional properties associated with the phenotype of M1 or M2 macrophages were determined.

Materials and methods

Culture and stimulation of J774 and BMDMS

Experiments were carried out using the J774.2 murine macrophage cell line originally derived from BALB/c mouse (J774; Sigma-Aldrich, St. Louis, MO, USA) or bone marrow (BM)-derived macrophage differentiated from BM isolated from BALB/c mice of both sexes, aged 8–12 weeks (AnLab, Prague, Czech Republic). BM was cultured in Dulbecco's Modified Eagle's Medium – high glucose (DMEM; Sigma-Aldrich) with 20% M-CSF-conditioned medium (M-CSF-conditioned medium was collected from L929 M-CSF cell line) for one week; afterwards, macrophages were used in experiments. J774 ($2{,}5\times10^5$ cells/ml) or BM-derived macrophages (5×10^5 cells/ml) were cultured in a volume of 1 ml DMEM supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), antibiotics (100 µg/ml streptomycin, 100 U/ml penicillin) and 10 mM HEPES buffer (hereafter called 'complete DMEM') in 24-well tissue culture plates (Nunc, Roskilde, Denmark). Capsaicin (10 µM; Sigma-Aldrich) was used for TRPV1-dependent activation as is routinely used [26, 27]. Lipopolysaccharide (LPS; 1,25 µg/ml; #L2880; Sigma-Aldrich) was used for the induction of immune responses. The concentrations and times of stimulation were tested and selected according to calibration and kinetic experiments. The preincubation was set for 4 h and the stimulation varied among methods. In detail, 5 minutes for the study of MAP kinases phosphorylation, 10 min for the translocation of ERK1/2 into the

nucleus, and 24 h for microscopy, PCR, ELISA, and flow cytometry (48 h for LIGHT, CD206, Mgl2 and CD163).

Immunostaining of J774

Stimulated J774 cells ($1,5\times10^3$ cells/ml) were cultured in 24-well tissue culture plate for 24 h. Cells were washed in PBS and fixed with 4% paraformaldehyde in PBS for 10 min, then washed in PBS and stained with Wheat Germ Agglutinin 647 (1:200; Thermo Fisher Scientific, Waltham, MA, USA) for 10 min at 37 °C. Subsequently, cells were washed in PBS, permeabilized with 0,1% Triton X-100® in PBS and blocked with 1% BSA and 10% donkey serum for 1 h at 37 °C. The cells were then washed in PBS and stained for 1 h at 37 °C with rabbit anti-mouse TRPV1 antibody (ACC-030, 1:200; Alomone Labs, Jerusalem, Israel), which has been validated using a KO mouse model [28]. After washing in PBS, cells were stained with the secondary donkey anti-rabbit IgG antibody Alexa Fluor Plus 488 (1:300; Thermo Fisher). Sample mounting with nuclei staining was performed using Ibidi mounting medium with DAPI (IBIDI, Gräfelfing, Germany). The samples were observed with Carl Zeiss LSM 880 NLO microscope (Zeiss, Oberkochen, Germany). Huygens deconvolution was performed on the high-resolution confocal images.

PCR

Total RNA was isolated from the cultured cells using TRIreagent® (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. Reverse transcription was performed with SuperScript™ IV Reverse Transcriptase (Thermo Fisher) according to the manufacturer's instructions, including RNaseOUT™ Recombinant Ribonuclease Inhibitor (Thermo Fisher) and Random Hexamer Primer (Thermo Fisher). RT-PCR was performed by PPP Master Mix (Top-Bio, Vestec, Czech Republic) according to the manufacturer's instructions. Quantitative PCR was performed by using HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) according to the manufacturer's instructions and measured by LightCyler480 (Roche, Basel, Switzerland). *Gapdh* and *Actb* were used as housekeeping genes. The primers are detailed in Supplementary Table S1.

SDS-page electrophoresis and western blot

J774 samples were sonicated and solubilized in Laemmli buffer. The proteins were separated using SDS-Page Electrophoresis and transferred to the nitrocellulose membrane. The membrane was blocked in 5% non-fat dry milk in TBS (10mM Tris, 150mM NaCl; pH 8.0) for 30 min. Specific primary antibodies were diluted in 1% non-fat dry milk or BSA in TBS. The nitrocellulose membrane was incubated with the following primary antibodies against extracellular signal-regulated kinase (ERK)1/2 (137F5; Cell Signaling, Danvers, MA, USA), p-ERK1/2 (197G2; Cell Signaling), p38 (sc-535; Santa Cruz, Dalas, TX, USA) or p-p38 (D3F9; Cell Signaling), and gently rocked at 4 °C overnight, followed by three 10 min washes in TBS containing 0,3% Tween-20. β-Actin (sc-47778, Santa Cruz) was used as a protein-loading control. Subsequently, the membrane was incubated with appropriate HRP-conjugated secondary antibodies for 1 h at RT and followed by three 10 min washes as mentioned above. Protein bands were visualized using enhanced chemiluminescence according to the manufacturer's instructions and developed using the ChemiDoc Imaging System (BioRad; USA). The developed images were analyzed using ImageLab software.

FLUO4-NW calcium measurement

Calcium influx was determined using FLUO4-NW dye (Thermo Fisher) according to the manufacturer's instructions. Briefly, J774 cells were seeded in the Poly-L Lysine coated 96 black well plate with a clear bottom. After 24 h, the dye was diluted in an assay buffer containing 2,5 mM Probenecid. Medium was removed, and cells were incubated with the dye for 30 min at 37 °C in a humified atmosphere with 5% CO_2. Calcium response was measured at 37 °C using the ClarioStar Plus microplate reader. Excitation was set at 494 nm and emission was measured at 516 (±30) nm. The signal was measured every 1 s. After 3 s of measurement, the appropriate ligand was automatically added by the machine using a pump. Data of the FLUO4 fluorescence changes were normalized to the baseline (before the addition of ligand) and calculated as a fold increase over the baseline.

ERK1/2 nuclear translocation

Cultured cells were washed in ice cold PBS, fixed (1% PFA, 20 min, RT), permeabilized (100% methanol, 15 min on ice) and stained with the following antibodies: anti-p44/42 MAPK (ERK1/2; 137F5; Cell Signaling); secondary antibody Goat anti-Rabbit IgG (A-11,012; Alexa Fluor 594; Thermo Fisher) at times and concentrations recommended by the manufacturer. For cell nuclei visualization, Hoechst 33258 fluorescent dye (Sigma-Aldrich) was used 5 min before measurement. Analysis of the samples was performed using the Amnis® ImageStream® Mk II imaging cytometer (Luminex Corporation, Austin, Tx, USA). The data obtained were then analyzed using Ideas software (Luminex Corporation) and its built-in module for the analysis of nuclear translocation. Briefly – after gating the cells based on their size and aspect ratio (singlets determination) and focus gradient, the shape of each cell and its nucleus was determined by the built-in algorithm. The correlation of Hoechst 33258 (nucleus) and AF 594 (ERK1/2) signal intensity within the cell

was evaluated, and the Similarity Median (SM) parameter was calculated. The area containing cells with signs of translocation was gated using the SM parameter, and cells within this gate were scored as „ERK translocated". Representative images of the cells, dot plots, and gates are shown in Supplementary Fig. S1.

Staining of reactive oxygen species, intracellular nitric oxide and membrane potential of mitochondria

J774 were stained for the detection of ROS production using 2′,7′-Dichlorofluorescin diacetate (DCFDA; 15 µM; Sigma-Aldrich), for intracellular nitric oxide (NO) using diaminofluorescein-2 diacetate (DAF-2 DA; 2,5 µM; Abcam, Cambridge, UK) or for mitochondrial membrane potential using MitoTracker™ Red CMXRos (100 nM; Thermo Fisher) according to the manufacturer's instructions for 30 min at 37 °C. Cells were harvested and washed in PBS/0.5% BSA. A total of 50 000 cells were analyzed after the exclusion of dead cells and debris. Five minutes before measurement, Hoechst 33258 fluorescent dye was added and used to exclude dead cells. Data were collected using the LSR II cytometer and analyzed using GateLogic 400.2 A software. Representative dot plots and histograms illustrating the gating strategy are shown in Supplementary Figure S2.

Characterization of surface markers by flow cytometry

Cultured cells were harvested and washed in PBS/0.5% BSA and incubated for 30 min on ice with Alexa Fluor 700 labeled anti-CD45 monoclonal antibody (mAb) (clone 30-F11; BioLegend), APC labeled anti-CD11b mAb (M1/70; BioLegend), FITC labeled anti-H-2Kb/H-2Db mAb (MHCI; 28-8-6, BioLegend), FITC labeled anti-I-A / I-E mAb (MHCII; M5/114.15.2, BioLegend), FITC labeled anti-CD80 mAb (16-10A1; BioLegend), PE labeled anti-CD86 mAb (PO3; BioLegend), PE/Cy7 labeled anti-CD301b mAb (URA-1; BioLegend) or PE labeled polyclonal Ab anti-TNFSF14 (LIGHT; Bioss Antibodies, Woburn, MA, USA). Unstained cells were used as controls. A total of 50 000 cells were analyzed after exclusion of dead cells and debris. Five minutes before measurement, Hoechst 33258 fluorescent dye (Sigma-Aldrich) was added and used to exclude dead cells. Data were collected using the LSR II cytometer (BD Bioscience, Franklin Lakes, NJ, USA) and analyzed using GateLogic 400.2 A software (Invai, Mentone, Australia). Representative dot plots and histograms illustrating the gating strategy are shown in Supplementary Fig. S3.

Functional test with J774 and T helper cells

Naive $CD4^+$ T helper cells were isolated from the spleen of BALB/c mice using FACS Aria II (BD Bioscience). Red blood cells were removed from the splenic suspension using ACK buffer and splenocytes were stained with Alexa Fluor 700 labeled anti-CD4 mAb (GK1.5; BioLegend), dead cells were excluded by propidium iodide (Exbio, Vestec, Czech Republic). J774 cells (2×10^4 cells/ml) were cultivated for 4 hours with or w/o stimulation. Then $CD4^+$ T helper cells (2×10^5 cells/ml) were added together with stimulation with LPS or capsaicin. After 72 h, the proliferation expression of Ki67 was detected using flow cytometry. Briefly, cells were harvested, washed with PBS/0.5% BSA and incubated for 30 min on ice with Alexa Fluor 700 labeled anti-CD4 mAb (GK1.5; BioLegend), APC labeled anti-CD11b mAb (M1/70; BioLegend) and LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit (Thermo Fisher). Cells were then fixed and permeabilized using a Foxp3 Staining Buffer Set (Thermo Fisher) according to manufacturer's instructions. Subsequently, cells were stained intracellularly for 30 min with PE labeled anti-Ki67 mAb (SolA15; Thermo Fisher). All events were analyzed after the exclusion of dead cells and debris. Data were collected using the LSR II cytometer and analyzed using GateLogic 400.2 A. Representative dot plots illustrating the gating strategy are shown in Supplementary Fig. S4.

Intracellular detection of cytokines and markers

To analyze intracellular cytokine production, Phorbol 12-Myristate 13-Acetate (PMA; 20 ng/ml; Sigma-Aldrich), ionomycin (500 ng/ml; Sigma-Aldrich) and Brefeldin A (5 µg/ml; Thermo Fisher) were added to the cultures for at least 4 h of the incubation period. Cells were harvested, washed in PBS/0,5% BSA and incubated for 30 min on ice with Alexa Fluor 700 labeled anti-CD45 mAb (clone 30-F11; BioLegend), APC labeled anti-CD11b mAb (M1/70; BioLegend) and LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit (Thermo Fisher) for staining dead cells. Cells were then fixed and permeabilized using a Fixation and Permeabilization Kit (Thermo Fisher) according to the manufacturer's instructions. Cells were then intracellularly stained for 30 min with PE labeled anti-TNFα mAb (TN3-19.12; Thermo Fisher), APC labeled anti-IL-6 mAb (MP5-20F3; BioLegend), FITC labeled anti-IL-1β Pro-form mAb (NJTEN3; Thermo Fisher), FITC labeled anti-CD206 mAb (C068C2; BioLegend) or PE labeled anti-CD163 mAb (TNKUPJ; Thermo Fisher). A total of 50 000 cells were analyzed after exclusion of dead cells and debris. Data were collected using the LSR II cytometer and analyzed using GateLogic 400.2 A software. Representative dot plots illustrating the gating strategy are shown in Supplementary Fig. S5.

ELISA cytokine detection

Cell supernatants were harvested after 24 h and the concentration of cytokines IL-1β, IL-6 and TNFα was

measured by ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Leishmania Cell Culture & Macrophage Infection

The promastigotes labelled with GFP *L. mexicana* (MNYC / BZ / 62 / M379) were cultured in M199 (Sigma-Aldrich) supplemented with 10% FBS, 1% BME vitamins (Sigma-Aldrich), 0.5% sterile human urine and 0.1% amikacin (Sigma-Aldrich) at 23 °C. The low passage of parasites was used for the experiments.

Stimulated J774 cells were cultured in complete DMEM medium on a 6-well tissue culture plate for 24 h. Cells were harvested and counted. 5×10^4 cells were plated in 12-well tissue culture plate in 2 ml complete DMEM and infected with *L. mexicana* promastigotes at a stationary phase of growth with the ratio of 6 parasite promastigotes per 1 macrophage. 72 h after infection, cells were analyzed by flow cytometry. The number of amastigotes was counted by hemocytometer as described [29]. For flow cytometry, cells were harvested and washed in PBS/0,5% BSA. All events were analyzed after the exclusion of dead cells and debris. 5 min before measurement, Hoechst 33258 fluorescent dye was added to exclude dead cells. Data were collected using the LSR II cytometer and analyzed using GateLogic 400.2 A software. Representative dot plots and histograms illustrating the gating strategy are shown in Supplementary Fig. S6.

Statistical analysis

For statistical analysis, the program The Prism (GraphPad Software, San Diego, CA, USA) was used. Data are shown as mean ± standard deviation (SD) or as boxes and whiskers. Lines inside the boxes represent the median, and whiskers represent the minimum and maximum values. In detail, 3 samples/group for nuclear translocation and for *L. mexicana* infection, 4 samples/group for ELISA, 5 samples/group for flow cytometry and qPCR, and 6 samples/group for western blot analysis. The statistical significance of differences between individual groups was calculated using ordinary One-Way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. Two-way ANOVA followed by Tukey's post hoc test was calculated for calcium measurement. A value of $p<0.05$ was considered statistically significant.

Results

TRPV1 expression in murine macrophages

To study capsaicin-induced changes in macrophage phenotype, the routinely used mouse cell line J774 and mouse BM-derived macrophages were selected [30]. The presence of TRPV1 was documented using confocal microscopy and TRPV1 mRNA expression by PCR analysis (shown in Fig. 1a, b). Full length gel. analysis of TRPV1 protein expression in the J774 cell line showed no change with the different stimulation settings (shown in Supplementary Fig. S7).

LPS pretreatment increases capsaicin-induced calcium influx

To determine whether LPS pretreatment affects the functional potential of TRPV1 in the J774 cell line, we analyzed capsaicin-induced calcium influx in cells pretreated with and without LPS by measuring the fluorescent intensity of FLUO4-NW dye. As shown in Fig. 2a, capsaicin induced an increase in intracellular calcium levels, which was enhanced in cells pretreated with LPS for 4 h.

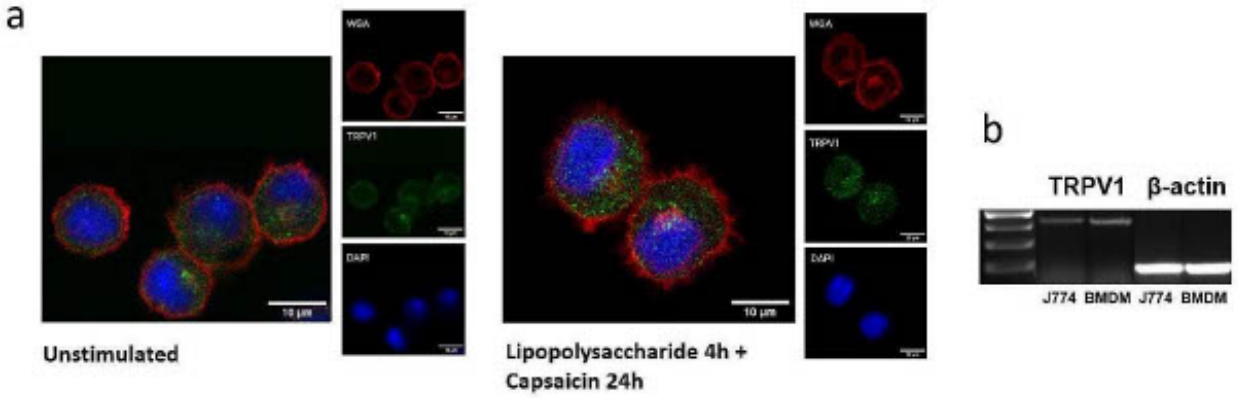


Fig. 1 Expression of TRPV1 in murine macrophages. TRPV1 expression was documented by immunohistochemical detection in J774 cells by confocal microscopy (red – WGA 647, green – TRPV1, blue – DAPI) (**a**). Expression of mRNA level in J774 cells and bone marrow-derived macrophages (BMDM) was documented by PCR (**b**). An uncropped image of the gel is shown in Supplementary Fig. S8

Capsaicin modulates LPS-induced signaling cascades in the J774 cell line

Activation of the TRPV1 channel increases intracellular Ca^{2+} levels and activates various protein kinases, including mitogen-activated protein kinases (MAPKs), particularly ERK and p38. These MAPKs are also associated with the M2 and M1 phenotype, respectively [31]. Western blot results showed that the p38 MAPK signaling pathway was not activated in response to capsaicin, and phosphorylation of p38 MAPK was associated only with LPS stimulation (shown in Fig. 2b). On the other hand, five minutes of capsaicin stimulation significantly increased p-ERK1/2 levels compared with unstimulated J774 cells. LPS treatment activated ERK1/2 in J774 cells, but this increase was not significant compared with the control cells. An increased level of p-ERK1/2 was detected five minutes after LPS stimulation and remained elevated four hours after LPS treatment (shown in Fig. 2c). While pretreatment of J774 cells with capsaicin or LPS promoted phosphorylation of ERK1/2 induced by LPS or capsaicin, the phosphorylated form of ERK1/2 was suppressed when cells were treated with LPS and capsaicin

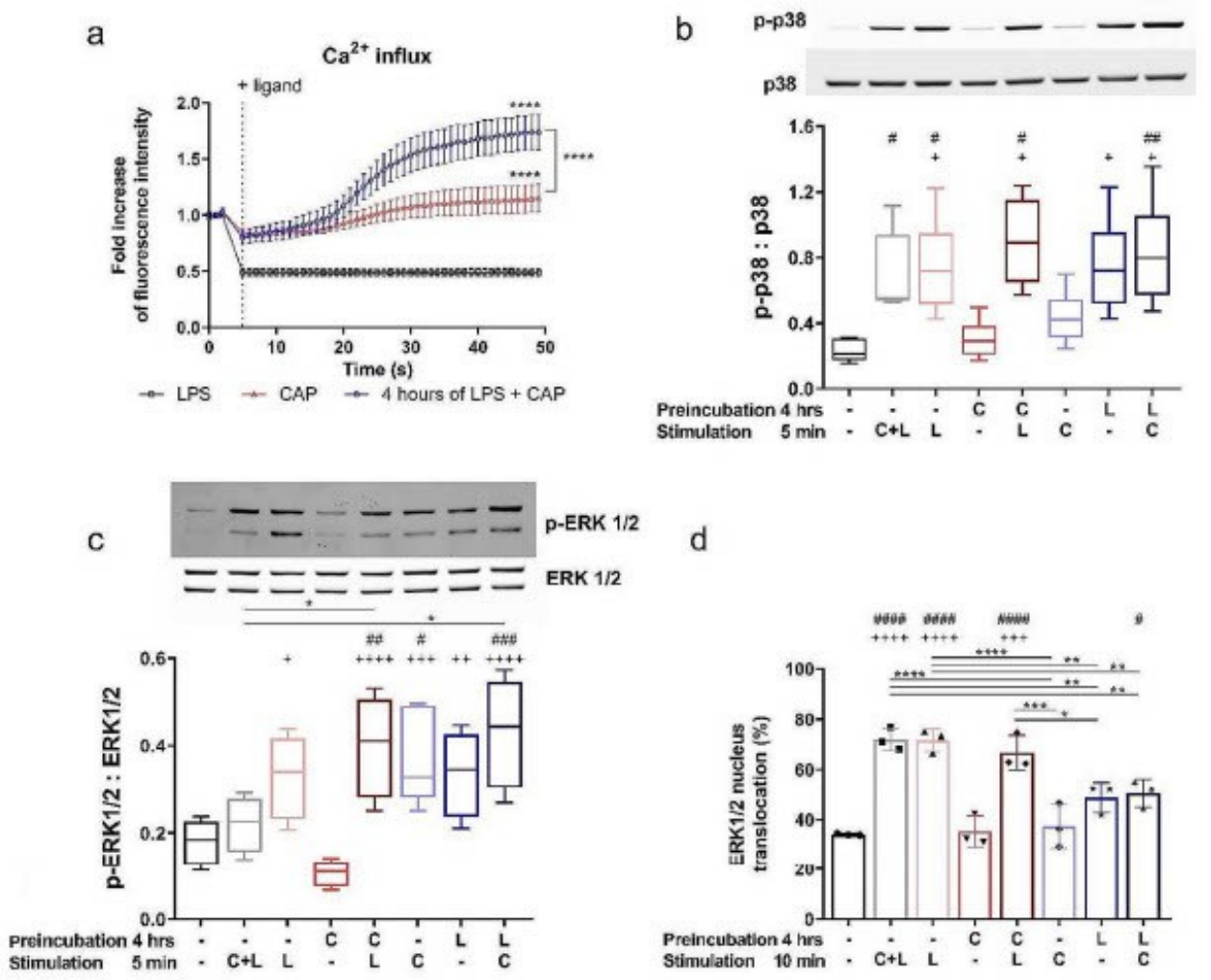


Fig. 2 Capsaicin-induced calcium influx and modulation of LPS-induced signaling cascades. The kinetics of capsaicin-induced calcium mobilization in J774 cells were measured by the fluorescence of FLUO4-NW (**a**). Data represent mean values ± SEM. The level of statistical significance was determined using 2-way ANOVA followed by Tukey's test for multiple comparisons (****$p < 0.0001$). For detection of LPS-induced signaling cascades J774 were preincubated for 4 h and stimulated with capsaicin (C) or LPS (L) for 5–10 min. The ratio between phosphorylated and unphosphorylated p-38 (**b**) and ERK 1/2 (**c**) was obtained by Western blotting after normalizing the intensities of the p-p38 and p-ERK1/2 bands to those of total p38 and ERK1/2, respectively. Uncropped images of the western blot membranes are also shown in Supplementary Fig. S9 and S10. Data are shown as boxes and whiskers. The lines within the boxes represent the median, and the whiskers represent the minimum and maximum values. $n = 6$ (**a-c**). Representative western blots are shown. The percentage of cells in which ERK1/2 translocated to the nucleus was determined by image flow cytometry. $n = 3$ (**d**). Data are shown as means ± SD. The level of statistical significance was determined using one-way ANOVA followed by Tukey's test for multiple comparisons ($^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$, $^{\wedge\wedge\wedge\wedge}p < 0.0001$). $^{\#}$ indicates the significance from (– –) group. $^{+}$ indicates significance from (C –) group. Other statistical significances between groups are indicated by *

together. Since activation of ERK1/2 signaling pathways can have a number of often contradictory effects that are cell-type and context-dependent. Several mechanisms are involved in deciding which signaling pathways are triggered, the most important of which is the cellular localization of phosphorylated ERK activity [32]. Therefore, we used Image stream flow cytometry to analyze ERK1/2 translocation into the nucleus. Interestingly, ERK1/2 nuclear translocation of each group did not correlate with ERK1/2 phosphorylation, indicating that various treatments may have different effects on the resulting macrophage phenotype (shown in Fig. 2d).

Capsaicin modulates LPS-induced ROS and NO production by the J774 cell line

Macrophage activation is associated with the generation of ROS, with both M1 and M2 macrophages showing the capacity to produce ROS after activation [33]. Therefore, we determined the intracellular level of ROS and NO in the J774 cell line stimulated with capsaicin simultaneously, prior or after LPS stimulation. Although capsaicin alone did not affect ROS and NO levels, their production was increased in LPS-stimulated cells. Notably, in cells treated with LPS and capsaicin together, or pretreated with capsaicin, the mean fluorescence intensity (MFI) of DCFDA and DAF-2 DA decreased significantly, indicating lower generation of ROS and NO, while cells pretreated with LPS prior to capsaicin stimulation showed an increase in MFI (shown in Fig. 3a, b). Another important factor associated with leukocyte activation is the increase in mitochondrial membrane potential [34]. LPS-pretreated cells exhibited the highest mitochondrial potential compared to the other groups, as indicated by an increase in MFI of MitoTracker Red CMXRos (shown in Fig. 3c).

Capsaicin modulates LPS-induced expression of MHC molecules and costimulatory receptors and the ability to stimulate T cells

Subsequently, we investigated whether an increase in ROS and NO production was associated with changes in the phenotype of J774 cells. MHC molecules, and the co-stimulatory molecules CD80 and CD86 are crucially involved in macrophage activation and antigen presentation during inflammation. Indeed, the expression of MHC I and II was significantly increased in cells pretreated with LPS as compared to LPS alone, LPS and capsaicin together, and pretreatment with capsaicin (shown in Fig. 4a, b). As shown in Fig. 4c, d, pretreatment with LPS also significantly enhanced the expression of the costimulatory molecules CD86 and CD80 compared to stimulation with LPS and capsaicin together and pretreatment with capsaicin. To determine whether the increased expression of molecules associated with antigen-presenting function correlated with the capacity to stimulate T cells, CD4 positive T cells were sorted and added to the J774 cell culture in all groups studied. Indeed, the expression of Ki67, a marker of proliferating cells, was highest in T cells in coculture with LPS-pretreated J774 cells, while pretreatment with capsaicin decreased the ability of J774 macrophages to provide the co-stimulatory signal to T cells (shown in Fig. 4e).

Capsaicin modulates LPS-induced cytokine production

Another important factor in determining the functional properties of macrophages is their production of

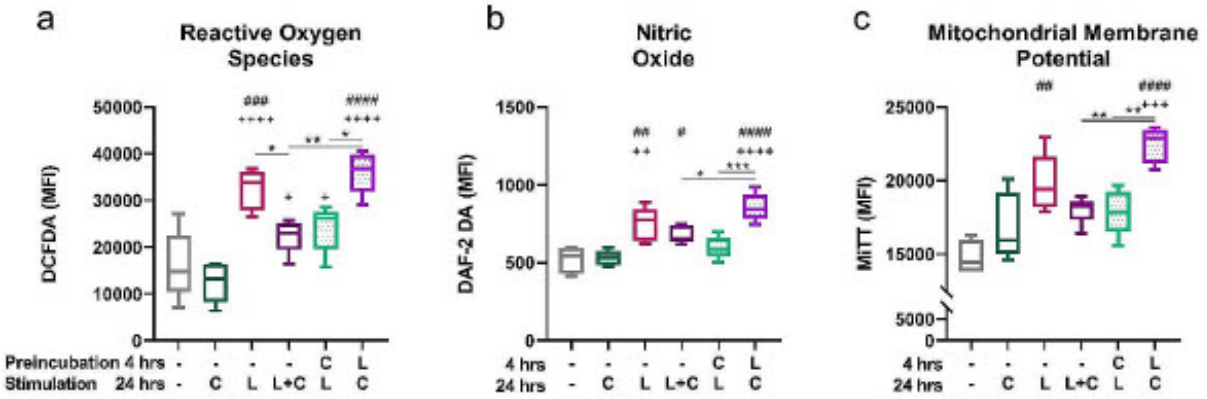


Fig. 3 LPS-induced generation of reactive oxygen species (ROS) and nitric oxide (NO) modulated by capsaicin. J774 cells were preincubated for 4 h and cultured with capsaicin (C) or LPS (L) for 24 h. Mean fluorescence intensity (MFI) of DCFDA for the presence of ROS (**a**), DAF-2 DA for NO (**b**) and MitoTracker Red CMXRos for mitochondrial membrane potential (**c**) were determined by flow cytometry. Data are shown as boxes and whiskers. Lines inside the boxes represent the median, and whiskers represent the minimum and maximum values. $n = 5$. The level of statistical significance was determined using one-way ANOVA followed by Tukey's test for multiple comparisons ($^{*}p < 0.05$, $^{nn}p < 0.01$, $^{nnn}p < 0.001$, $^{nnnn}p < 0.0001$). # indicates significance from (– –) group. + indicates significance from (– C) group. Other statistical significances between groups are indicated by *

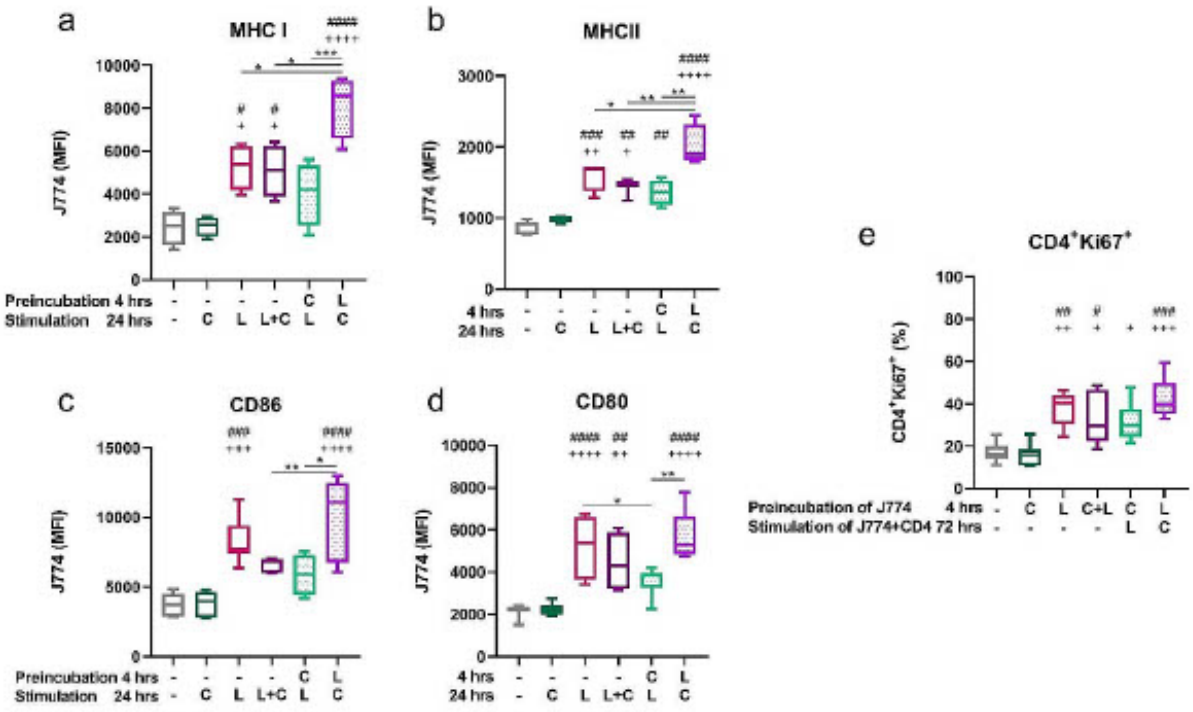


Fig. 4 The effect of capsaicin on LPS-induced expression of costimulatory receptors and the capacity to stimulate T cells. J774 cells were preincubated for 4 h and cultured with capsaicin (C) or LPS (L) for 24 h. The MFI of MHC I (**a**) or MHC II (**b**), CD86 (c), and CD80 (**d**) of J774 was determined by flow cytometry. (**e**) The proliferation capacity of $CD4^+$ T cells, was determined, according to the Ki67 expression, by flow cytometry. J774 were preincubated for 4 h with capsaicin or LPS. Afterwards, J744 and $CD4^+$ (in a 1:20 ratio) were co-cultivated for 72 h in presence of capsaicin or LPS. Data are shown as boxes and whiskers. Lines inside the boxes represent the median, and whiskers represent the minimum and maximum values. $n = 5$. The level of statistical significance was determined using one-way ANOVA followed by Tukey's test for multiple comparisons ($^{n}p < 0.05$, $^{nn}p < 0.01$, $^{nnn}p < 0.001$, $^{nnnn}p < 0.0001$). $^{\#}$ indicates significance from (– –) group. $^{+}$ indicates significance from (– C) group. Other statistical significances between groups are indicated by *

cytokines. LPS stimulation of J774 cells resulted in significant expression of genes for the pro-inflammatory cytokines TNFα, IL-6 and IL-1β, which were suppressed in LPS-pretreated cells (shown in Fig. 5a, b, c). On the contrary, while the presence of LPS in the culture medium suppressed the expression of the IL-10 gene, the LPS-pretreated group showed similar expression to the control group (shown in Fig. 5d). To correlate the amount of mRNA with protein production, cytokines released into culture medium were determined by ELISA. Capsaicin treatment in all combinations reduced the concentration of TNFα in the tissue culture medium compared to the group cultured with LPS alone, but interestingly, the level of IL-1β remained similar in all groups (shown in Fig. 5e, f). IL-6 concentration was increased in LPS-stimulated cells, with both pretreatments reducing its production, although significance was not reached (shown in Fig. 5g).

Capsaicin modulates the phenotype of bone marrow-derived macrophages

Since the response of primary macrophage can differ from that of macrophage cell lines [30, 35], we also tested the effect of capsaicin on BM-derived macrophages. As shown in Fig. 6a, b, CD86 expression was most pronounced in the LPS-pretreated group; however, the MHC II expression was similar in all groups. M2 markers were not detectable in the J774 cell line; however, the M2b marker TNFSF14, also known as LIGHT (homologous to lymphotoxin, inducible expression, competes with herpes simplex virus [HSV] glycoprotein D for HSV entry mediator, a receptor expressed on T lymphocytes), but not the M2a and M2c markers CD206, CD163 and CD301b (shown in Supplementary Fig. S11), increased on bone marrow-derived macrophages (shown in Fig. 6c). Consistent with changes indicating the macrophage switch into an anti-inflammatory phenotype, intracellular levels of inflammatory cytokines (IL-1β, TNF-α, IL-6) decreased significantly in LPS-pretreated primary macrophages (shown in Fig. 6d, e, f).

Capsaicin modulates the susceptibility of macrophages to *Leishmania mexicana*

Some myeloid cells serve as replication niches for the protozoan parasite *Leishmania*, and macrophages play

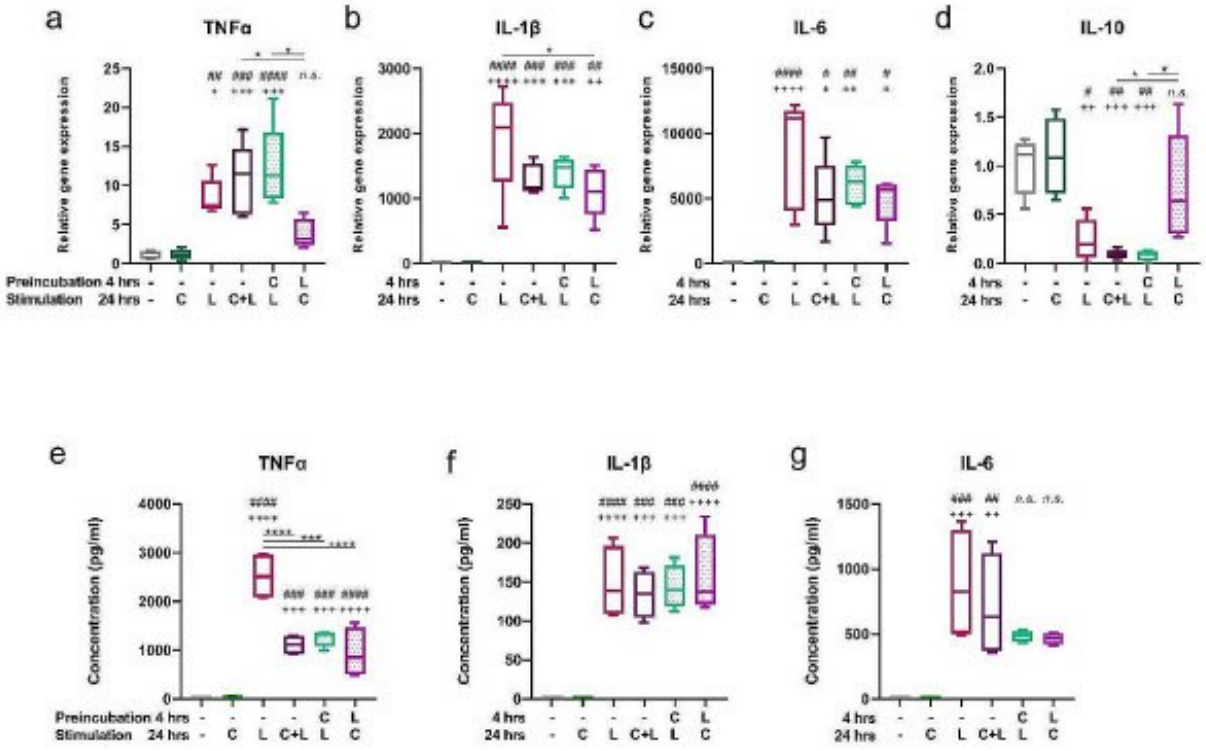


Fig. 5 Capsaicin modulates LPS-induced cytokine production. J774 were preincubated for 4 h and cultured with capsaicin (C) or LPS (L) for 24 h. The relative gene expression of TNFα (**a**), IL-1β (**b**), IL-6 (**c**) and IL-10 (**d**) was determined by qPCR. The concentration of TNFα (**e**), IL-1β (**f**) and IL-6 (**g**) in the supernatant was determined by ELISA. Data are shown as boxes and whiskers. The lines within the boxes represent the median and whiskers represent the minimum and maximum values (**a**–**g**). $n = 5$ (**a**–**d**), $n = 4$ (**e**–**g**). The level of statistical significance was determined using one-way ANOVA followed by Tukey's test for multiple comparisons ($^{n}p < 0.05$, $^{nn}p < 0.01$, $^{nnn}p < 0.001$, $^{nnnn}p < 0.0001$). $^{\#}$ indicates significance from (– –) group. $^{+}$ indicates significance from (– C) group. Other statistical significances between groups are indicated by *

a key role in the pathology of leishmaniasis. Individual subpopulations of macrophages contribute differently to the development of leishmaniasis, with macrophage susceptibility to *Leishmania* linked to the expression of certain M2 markers [36]. To further investigate whether capsaicin treatment affects macrophage phenotype, J774 cells were exposed to GFP-expressing *L. mexicana* [37] at a ratio of 6 parasites per 1 macrophage. Three days after infection, the percentage of *L. mexicana*-positive cells was the highest in LPS-pretreated cells (shown in Fig. 7a). In addition to different susceptibility to infection, the M2 phenotype has also been associated with the proliferation of amastigotes [36]. To address this question, the ratio of live amastigotes to the number of live cells in culture was compared between groups. The results shown in Fig. 7b indicate different proliferation of amastigotes in capsaicin- or LPS-pretreated groups, with the highest proliferation in LPS-treated cells and cells pretreated with LPS prior to capsaicin stimulation. This was associated with the highest mortality of *L. mexicana*-infected cells in this group (shown in Fig. 7c).

Discussion

Besides its role in nociception, TRPV1 is involved in various physiological functions. Currently, the role of TRPV1 in the regulation of immune response is gaining more attention, as it has been associated with several pathological conditions [38, 39]. Rapid progress in TRPV1 channel research has provided a better understanding of its action; however, it has also produced contradictory results on the inflammatory or anti-inflammatory TRPV1 function [2, 16, 17, 22]. To explore capsaicin triggered modulation of macrophage phenotype and function by capsaicin in different inflammatory environments, we activated the J774 cell line or primary BM-derived macrophages with capsaicin simultaneously, prior to or after LPS stimulation.

Macrophages display considerable differences in their response to inflammatory stimuli depending on their origin [35], including the expression of surface molecules and cytokine production [30, 40]. Therefore, we decided to study a macrophage cell line as well as macrophages derived from the bone marrow. Indeed, some results differ in macrophages of different origins; nevertheless,

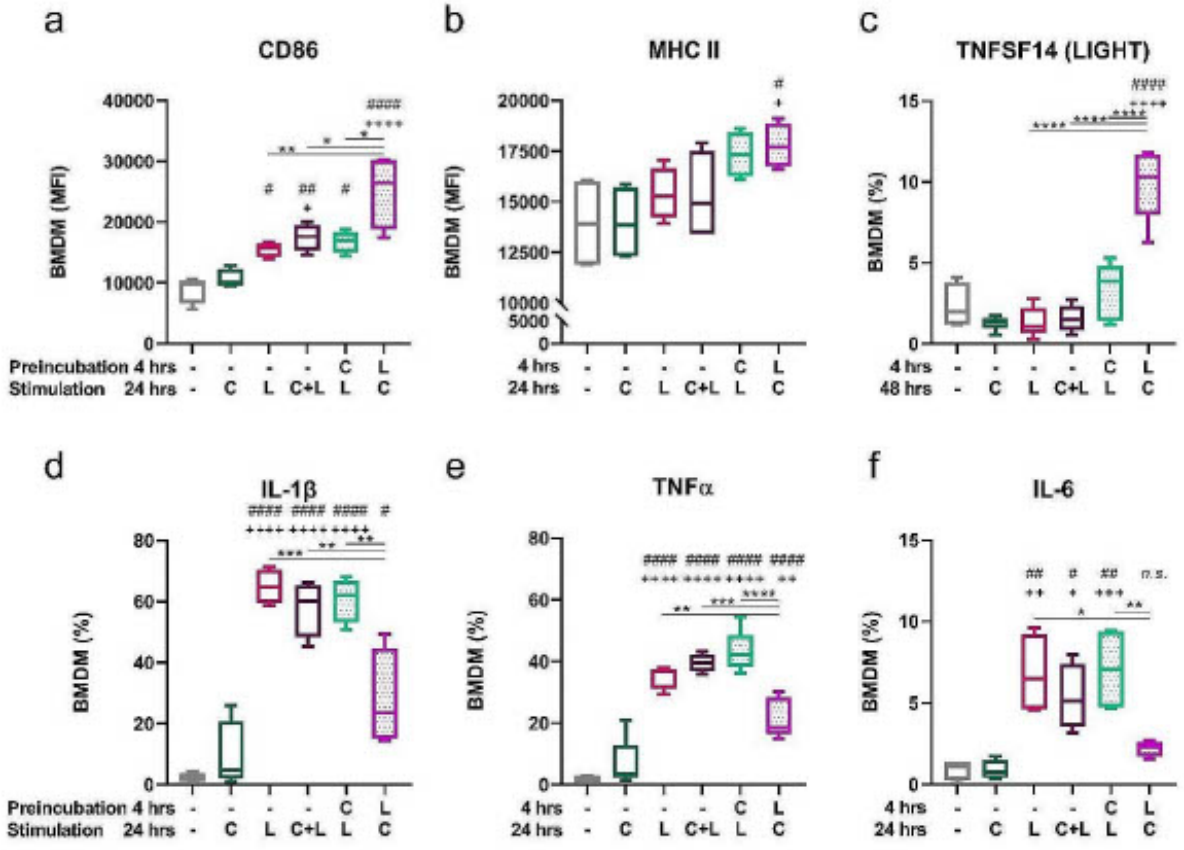


Fig. 6 The expression of surface markers and cytokines on BM-derived macrophages. Cells were preincubated for 4 h and cultured with capsaicin (C) or LPS (L) for 24 h (48 h for TNFSF14). MFI of CD86 (**a**) or MHCII (**b**) from BM-derived macrophages and the proportion of TNFSF14$^+$ (**c**), IL-1β$^+$ (**d**), TNFα$^+$ (**e**) and IL-6$^+$ (**f**) was determined by flow cytometry. Data are shown as boxes and whiskers. Lines inside the boxes represent the median, and whiskers represent the minimum and maximum values. $n = 5$. The level of statistical significance was determined using one-way ANOVA followed by Tukey's test for multiple comparisons ($^{n}p < 0.05$, $^{nn}p < 0.01$, $^{nnn}p < 0.001$, $^{nnnn}p < 0.0001$). $^{\#}$ indicates significance from (– –) group. $^{+}$ indicates significance from (– C) group. Other statistical significances between groups are indicated by *

combining the two types of macrophages provides a more comprehensive understanding of the role of TRPV1 in macrophage function and its inflammatory response. Importantly, both cell types express TRPV1 at mRNA and protein level which was confirmed by western blot and microscopy, using well validated antibodies [28]; therefore, providing a suitable tool for this study.

Different members of the MAPK family play distinct roles in modulating macrophage phenotype [31]. We confirmed that ERK1/2 signaling is involved in the TRPV1-mediated immune response; however, the level of phosphorylated ERK1/2 did not correlate with its translocation to the nucleus. ERK1/2 signaling has been shown to play a crucial role in many cellular processes, including proliferation, differentiation, or apoptosis, and is highly context-dependent [41]. Overall, cellular localization of activated ERK1/2 is a critical determinant of downstream signaling events; however, the effects of cellular translocation on the immune environment or cytokine production have not yet been described. Therefore, conflicting results that describe the up- or down-regulation of pro- and anti-inflammatory cytokines after ERK phosphorylation [41, 42] may be related to the differential cellular localization.

The finding that Ca^{2+} influx increased when macrophages were pretreated with LPS prior to activation with capsaicin indicated that the TRPV1 channel was sensitized by TLR4 stimulation. Sensitization of TRPV1 by LPS has been observed in sensory neurons, with the underlying mechanism involving inhibition of TLR4 activation-induced TRPV1 endocytosis [43]. Considering the expression of TLR4 on macrophages, it is reasonable to speculate that TLR4-TRPV1 interaction can occur here. The increased Ca^{2+} influx in the LPS-pretreated group was accompanied by an increase in the levels of ROS, NO and mitochondrial membrane potential, indicating that the overall macrophage activation state was increased. The increase in mitochondrial potential associated with

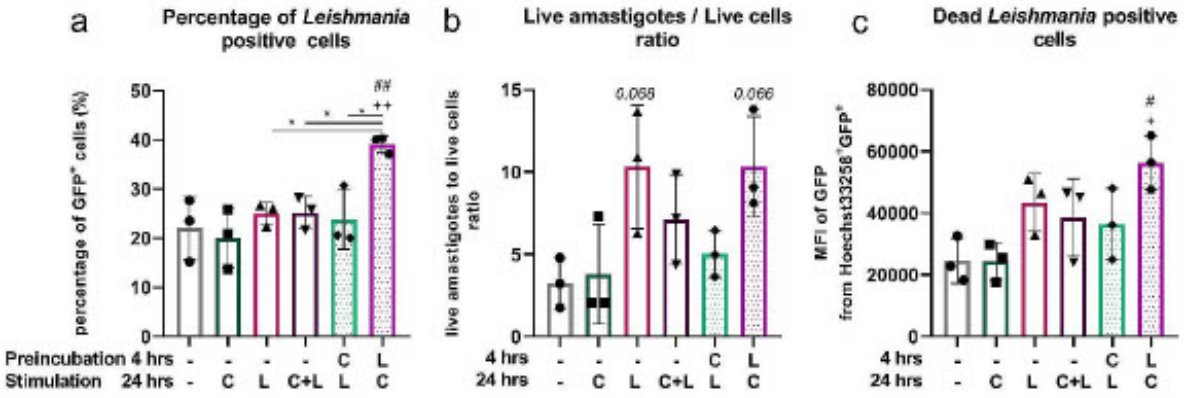


Fig. 7 Capsaicin modulates susceptibility of J774 to *Leishmania mexicana*. J774 cells were preincubated for 4 h and cultured with capsaicin (C) or LPS (L) for 24 h. GFP-labelled *L. mexicana* promastigotes were added to cells at a ratio of 6 parasites to 1 macrophage. Percentage of *Leishmania* positive live cells was determined by flow cytometry after 72 h incubation (**a**). The ratio of live amatigotes to the number of live cells was determined by hemocytometer (**b**). MFI of GFP-labelled *L. mexicana* from dead Hoechst33258$^+$ cells was determined by flow cytometry (**c**). Data are shown as means ± SD, $n = 3$ (**a**–**c**). The level of statistical significance was determined using one-way ANOVA followed by Tukey's test for multiple comparisons ($^*p < 0.05$, $^{**}p < 0.01$). $^\#$ indicates significance from (– –) group. $^+$ indicates significance from (– C) group. Other statistical significances between groups are indicated by *

the ROS release has been shown to play an essential role in several innate immune functions in both M1 and M2 macrophages, by regulating cell growth, differentiation, and apoptosis [44]. NO production is typically associated with M1 macrophages; nonetheless, the M2b phenotype is distinct from other M2 phenotypes due to its higher activity of iNOS and subsequent production of NO [45]. Moreover, the TRPV1 channel has been implicated in the regulation of NO release by the mechanisms mediated by the activity of NOS enzymes [46, 47]. Additionally, treatment with the NOS inhibitor L-NAME reveals the involvement of additional mechanisms. Torres-Narváez et al. (2019) proposed that capsaicin activates eNOS via $Ca2^+$ influx and PI3-kinase/Akt pathways, with L-NAME selectively blocking the former [48]. Clark et al. (2007) found increased NO production in TRPV1 KO mice, suggesting that TRPV1 negatively regulates NO production upon LPS stimulation [47]. Given the known suppression of inflammatory cytokine production by NO-mediated mechanisms, NO likely plays an immunoregulatory role in the resolution of inflammation [49]. Our data suggest that the timing of TRPV1 activation in relation to LPS is critical to this phenomenon.

The definition of the different macrophage phenotypes is based on the production of specific factors, expression of cell surface markers, and biological activities. The expression of markers and the production of factors associated with M1 or M2 activation can vary depending on the stimulus and context [50]. In our study, cells stimulated with LPS prior to TRPV1 activation expressed significantly higher levels of MHC I and II as well as co-stimulatory molecules. LPS pretreated macrophages also showed the best antigen presenting function when co-cultured with naïve T cells compared with the other groups. Although M1 macrophages are generally considered to have a stronger antigen-presenting ability than M2 macrophages, the M2b subset is an exception to this rule due to the expression of co-stimulatory molecules such as CD86 and MHC class II [45, 51]. TNFSF14, which is widely accepted as an M2b marker [52], was not expressed on the J774 cell line; however, activation of TRPV1 significantly increased its expression on BM-derived macrophages pretreated with LPS. Another important feature of M2b macrophages is the reduced production of inflammatory cytokines [51]. We detected decreased expression of TNFα at the both gene and protein levels in LPS-pretreated J774 cells, whereas the expression of IL-1β remained without significant changes. It should be noted that the intracellular level of TNFα, IL-1β and IL-6 was significantly decreased in primary macrophages when they were stimulated with LPS prior to capsaicin. Significant increase in anti-inflammatory cytokines was not detected in either J774 cells or in BM-derived macrophages. According to the review by Bujak et al. (2019), decreased production of inflammatory cytokines was observed after TRPV1 activation in various in vitro models of LPS-induced inflammation [53]. However, to our knowledge, no studies have demonstrated a switch to anti-inflammatory macrophage populations producing high levels of IL-10 and low levels of inflammatory cytokines after the TRPV1 activation. Stimulation of macrophages with capsaicin prior to LPS-induced inflammation did not suppress the release of inflammatory cytokines, not only in our model but also in other studies [54]. Thus, we propose that the production of pro-inflammatory cytokines is regulated by

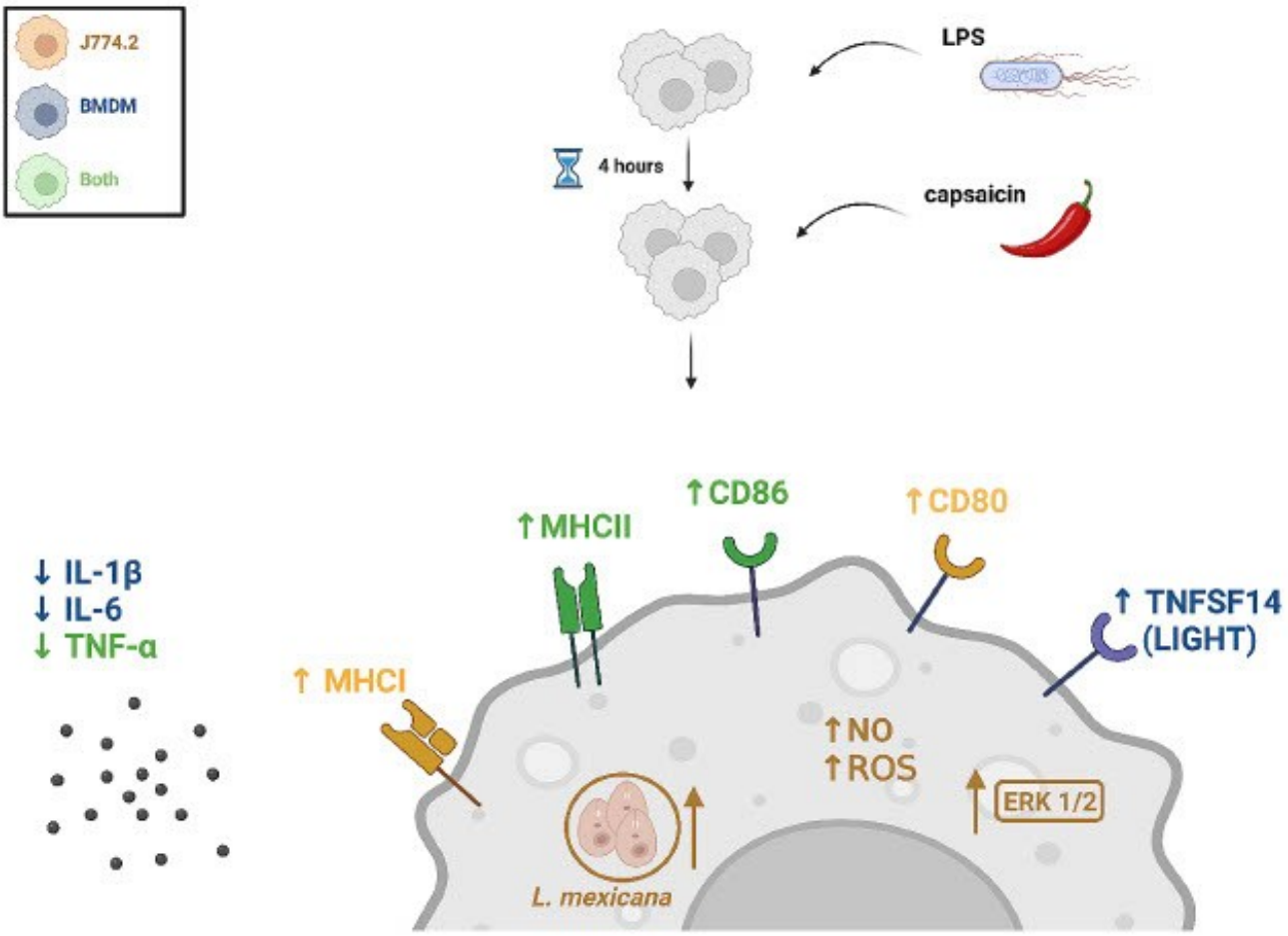


Fig. 8 Schematic illustration of the results of this study. Macrophages pretreated with LPS and subsequently treated with capsaicin possess unique properties of M2b-like regulatory polarization state. Created with Biorender.com

TRPV1 activation in a manner dependent on the inflammatory milieu.

To confirm the effect of TRPV1 activation after LPS stimulation on macrophage polarization state in the functional study, we investigated the survival of GFP-labelled *L. mexicana*, an intracellular parasite that naturally infects macrophages. Polarization of macrophages has been shown to affect the course of parasitic infection [55]. In our study, LPS pretreatment of capsaicin-stimulated macrophages led to a lower level of pro-inflammatory cytokines, which can potentially create a more permissive environment for the survival and replication of *L. mexicana* within the macrophages [55]. While the sample size in our study ($n=3$) can be considered limited, the infectious nature of the experiments presented challenges to achieving a larger sample size. Despite this limitation, our results are consistent and we believe they provide valuable insight into the survival and infection of the Leishmania parasite in macrophages. The fact that LPS treatment prior to capsaicin stimulation resulted in a higher percentage of infected macrophages, a trend toward a higher number of amastigotes per cell, and increased GFP signal intensity in dead macrophages supports our hypothesis that polarization of macrophages after TRPV1 activation is regulated by the immune microenvironment, which may also play a critical role in the outcome of parasitic infections.

Conclusions

In conclusion, our study shows that TRPV1 activation has specific effects on macrophages depending on the inflammatory environment, as summarized in Fig. 8. Stimulation with capsaicin of LPS-pretreated macrophages resulted in enhanced calcium influx and cell activation accompanied by a switch to the anti-inflammatory M2b-like polarization state, which was characterized by upregulated expression of MHC and co-stimulatory molecules and downregulated expression of inflammatory cytokines. ERK1/2 phosphorylation but not nuclear translocation was involved in this process. This study sheds light on the immunomodulatory mechanisms of capsaicin stimulation of macrophages in an inflammatory environment. The findings suggest that TRPV1 activation may have therapeutic potential for the treatment of

inflammatory diseases by promoting an anti-inflammatory response in macrophages.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12950-024-00391-0.

Supplementary Material 1

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Not applicable.

Author contributions
D.V., B.M., and M.K. designed the experiments. D.V., N.F., V.N.M., O.H., L.P., B.P., and V.S. performed experiments. D.V., N.F., V.N.M. analyzed and visualized the data. D.V. and M.K. prepared the manuscript. B.M., J.N., and M.K. critically revised the article and supervised the research. All the authors read and revised the final version of this article.

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Data availability
All data generated or analysed during this study are included in this article and its supplementary material files. Further enquiries can be directed to the corresponding author.

Declarations

Ethics approval and consent to participate
This study was carried out in strict accordance with the Act No. 246/1992 coll., on the protection of animals against cruelty, the basic law related to animal protection governing the activities of all the state authorities of animal protection in the Czech Republic. This study protocol was reviewed and approved by The expert commission for ensuring the welfare of experimental animals, Faculty of Science, Charles University approval number MSMT-31167/2021-4.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Additional file 1: Supplementary Material

Lipopolysaccharide pretreatment increases the sensitivity of the TRPV1 channel and promotes an anti-inflammatory phenotype of capsaicin-activated macrophages

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(1 Table and 11 Figures)

Table S1 of used primers for RT-PCR and q-PCR.

M. musculus **gene symbol**	**Official Full Name**	***M. musculus*** **protein symbol**	**NCBI Gene**	**Primer sequence**	**Use**
Trpv1	transient receptor potential cation channel, subfamily V, member 1	TRPV1	193034	GGGTCATTTCTCCCCTACGC CGTAGCAACACCAGCCCAA	RT-PCR
Actb	actin, beta	β-actin	11461	CATCCGTAAAGACCTCTATGCCAAC ATGGAGCCACCGATCCACA	RT-PCR
Il1b	interleukin 1 beta	IL-1β	16176	AGTTGACGGACCCCAAAAG AGTTGACGGACCCCAAAAG	q-PCR
Tnf	tumor necrosis factor	TNFα	21926	GCTCCAGTGAATTCGGAAAG GATTATGGCTCAGGGTCCAA	q-PCR
Il6	interleukin 6	IL-6	16193	GCTACCAAACTGGATATAATCAGGA CCAGGTAGCTATGGTACTCCAGAA	q-PCR
Il10	interleukin 10	IL-10	16153	AGCCGGGAAGACAATAACTG CATTTCCGATAAGGCTTGG	q-PCR
Gapdh	glyceraldehyde-3-phosphate dehydrogenase	GAPDH	16154	CGAGGATGGGAATAACTACTG GGATGATGA AGACAGCCTTGAAGTC	q-PCR
Actb	actin, beta	β-actin	11461	CATCCGTAAAGACCTCTATGCCAAC ATGGAGCCACCGATCCACA	q-PCR

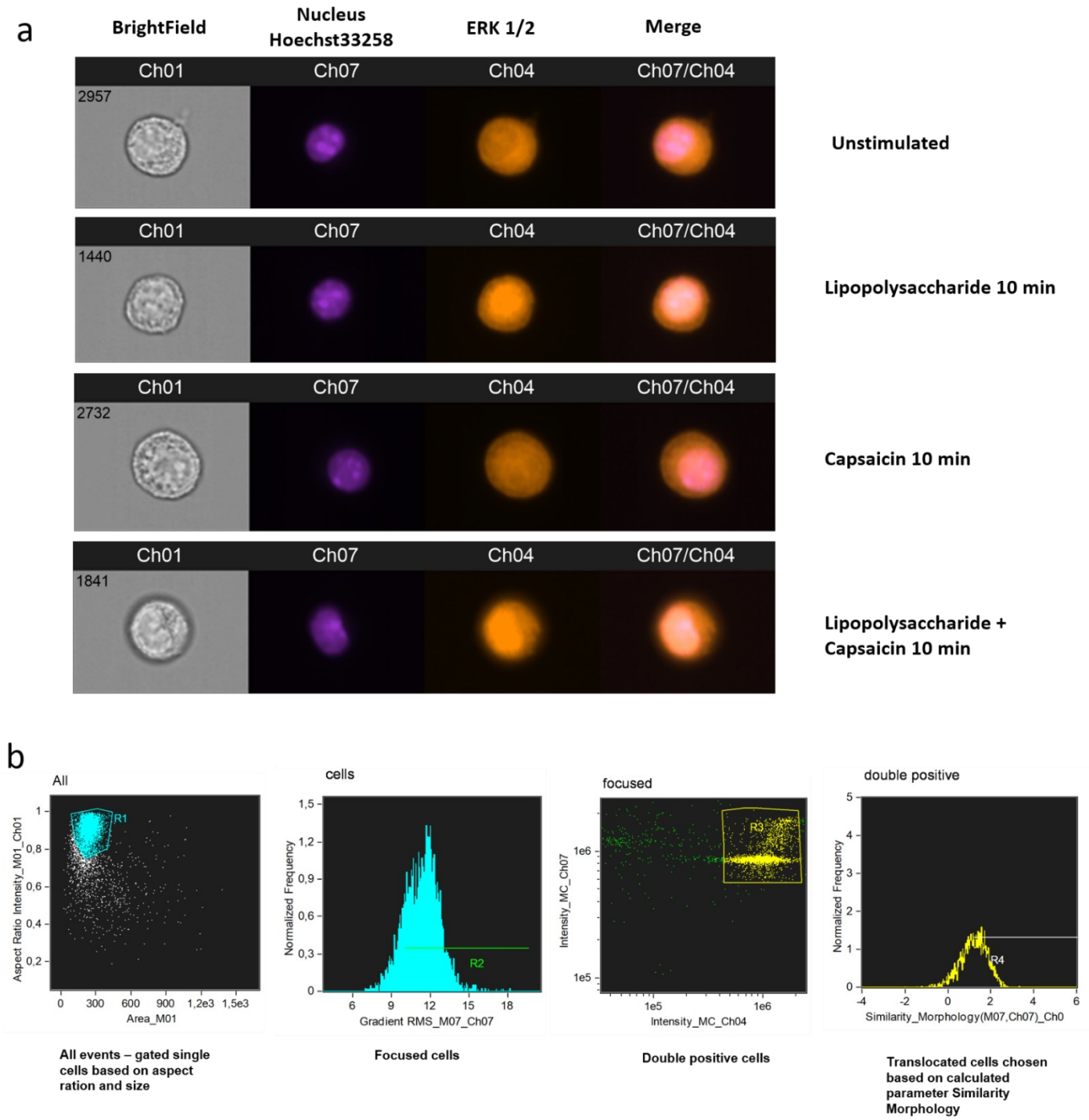


Figure S1 Gating strategy for ERK 1/2 translocation. J774 cells were preincubated for 4 hours and simulated for 10 minutes with capsaicin or/and lipopolysaccharide. Afterwards, translocation to the nuclei was determined by ImageStream flow cytometry. Example of acquired signals for individual cells (**a**). Gating strategy: after gating cells based on its size and aspect ratio (singlets determination) and focus gradient, shape of each cell and its nucleus has been determined by build-in algorithm and correlation of Hoechst 33258 signal intensity (nucleus) and AF 594 (ERK1/2) within the cell has been evaluated and Similarity Median (SM) parameter has been calculated. Region containing cells with signs of translocation has been gated on SM parameter and cells within this gate has been evaluated as „ERK translocated" (**b**).

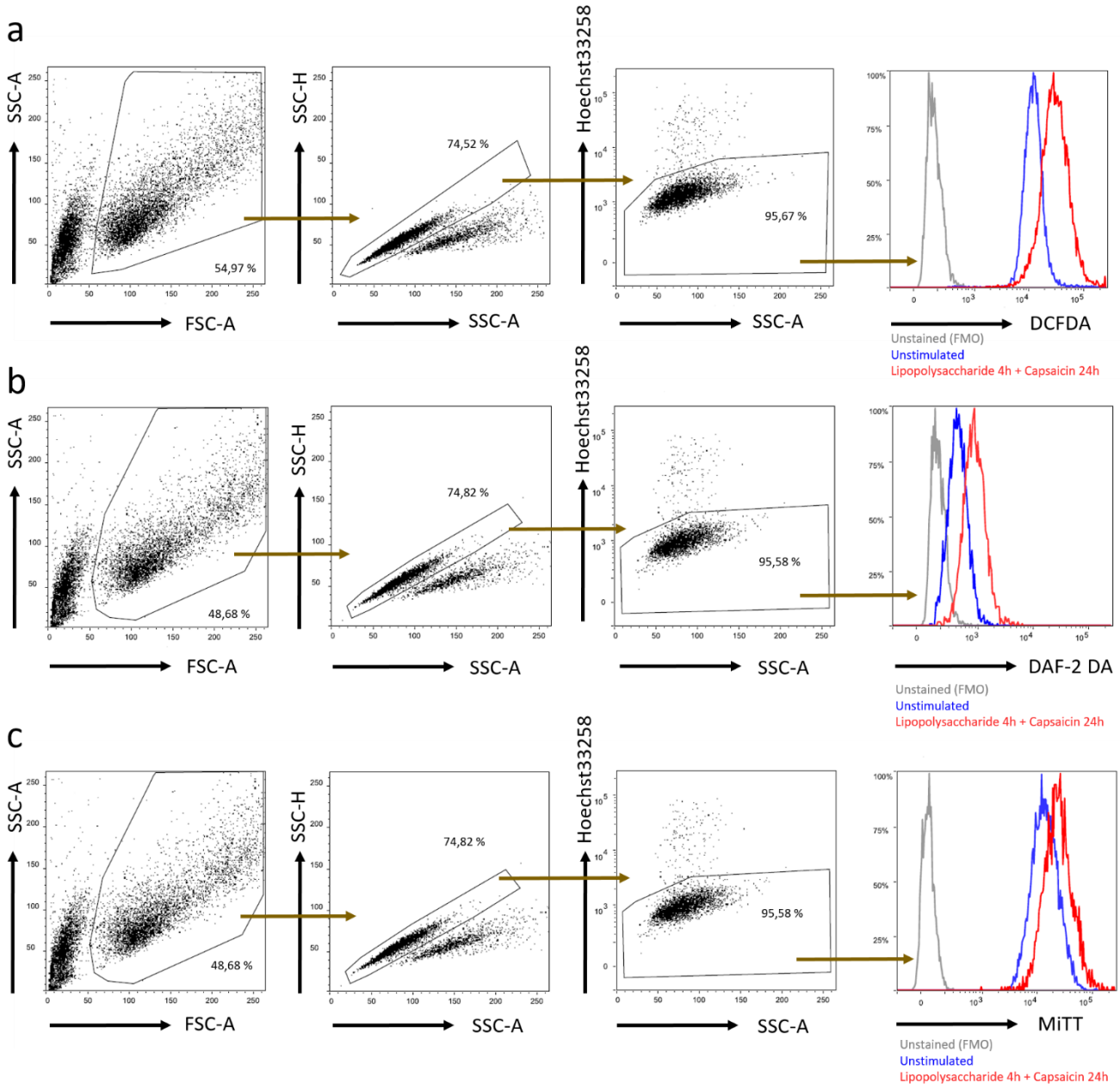


Figure S2 Gating strategy for Reactive Oxygen Species, Intracellular Nitric Oxide and Membrane Potential of Mitochondria Staining. J774 cells were preincubated for 4 hours and simulated for 24 hours with capsaicin or/and lipopolysaccharide. Afterwards, cells were intracellularly stained for the detection of ROS production using 2',7'-Dichlorofluorescin diacetate (DCFDA, **a**), for intracellular nitric oxide (NO) using diaminofluorescein-2 diacetate (DAF-2 DA, **b**) and for mitochondria membrane potential using MitoTracker Red CMXRos (MiTT, **c**). Gating strategy: debris was excluded based on forward and side scatter (FSC, SSC), then doublets were excluded and live were gated based on Hoechst 33258 negative population (live). From live J774 cells were determined mean fluorescent intensity for DCFDA, DAF-2 DA and MiTT. Grey – unstained FMO control, blue – unstimulated cells, red – cells preincubated with LPS 4 hrs and stimulated with capsaicin 24 hrs.

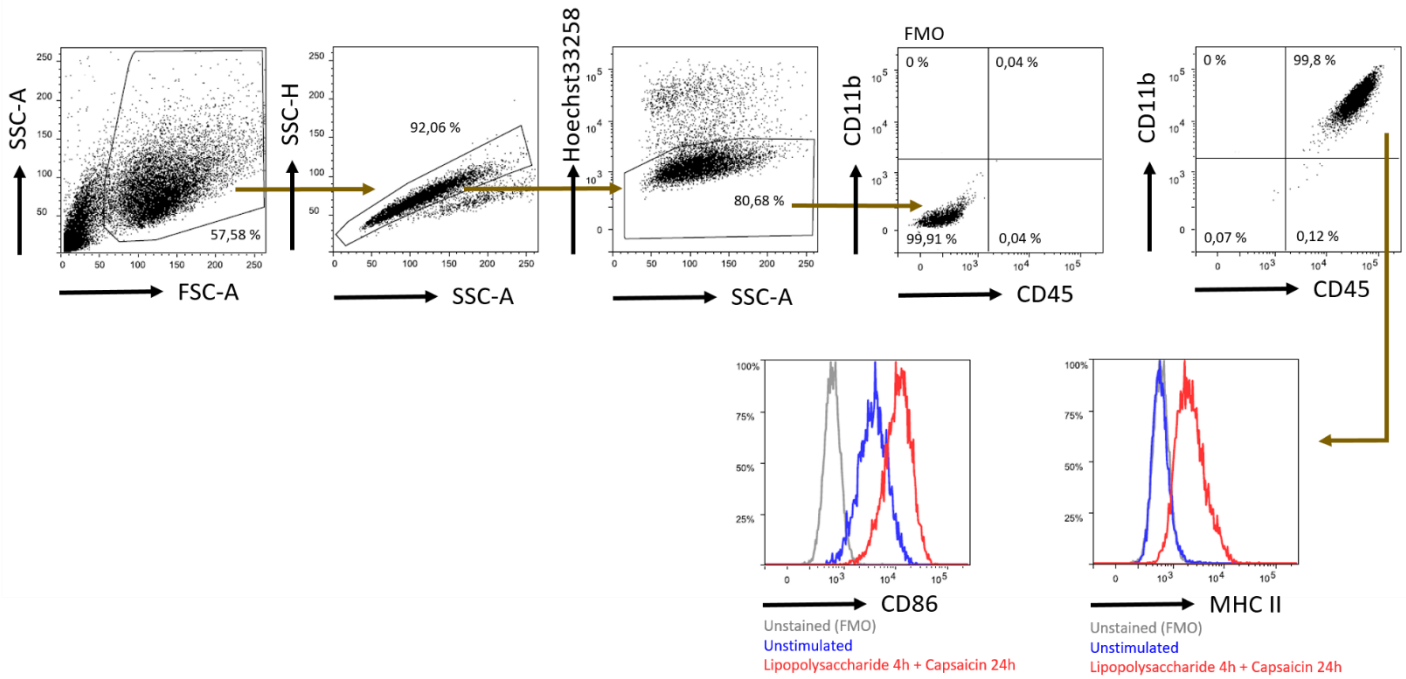


Figure S3 Gating strategy for surface markers. J774 or BMDM were preincubated for 4 hours and simulated for 24 hours with capsaicin or/and lipopolysaccharide. Afterwards, expression of surface markers was determined by flow cytometry. Gating strategy: debris was excluded based on forward and side scatter (FSC, SSC), then doublets were excluded, and live cells were gated based on Hoechst 33258 negative population (live). Macrophages were selected based on CD45 and CD11b positivity. From $CD45^{+}CD11b^{+}$ cells were determined mean fluorescent intensity for CD80, CD86, MHC I, MHC II and percentage of TNFSF14 positive cells. Grey – unstained FMO control, blue – unstimulated cells, red – cells preincubated with LPS 4 hrs and stimulated with capsaicin 24 hrs.

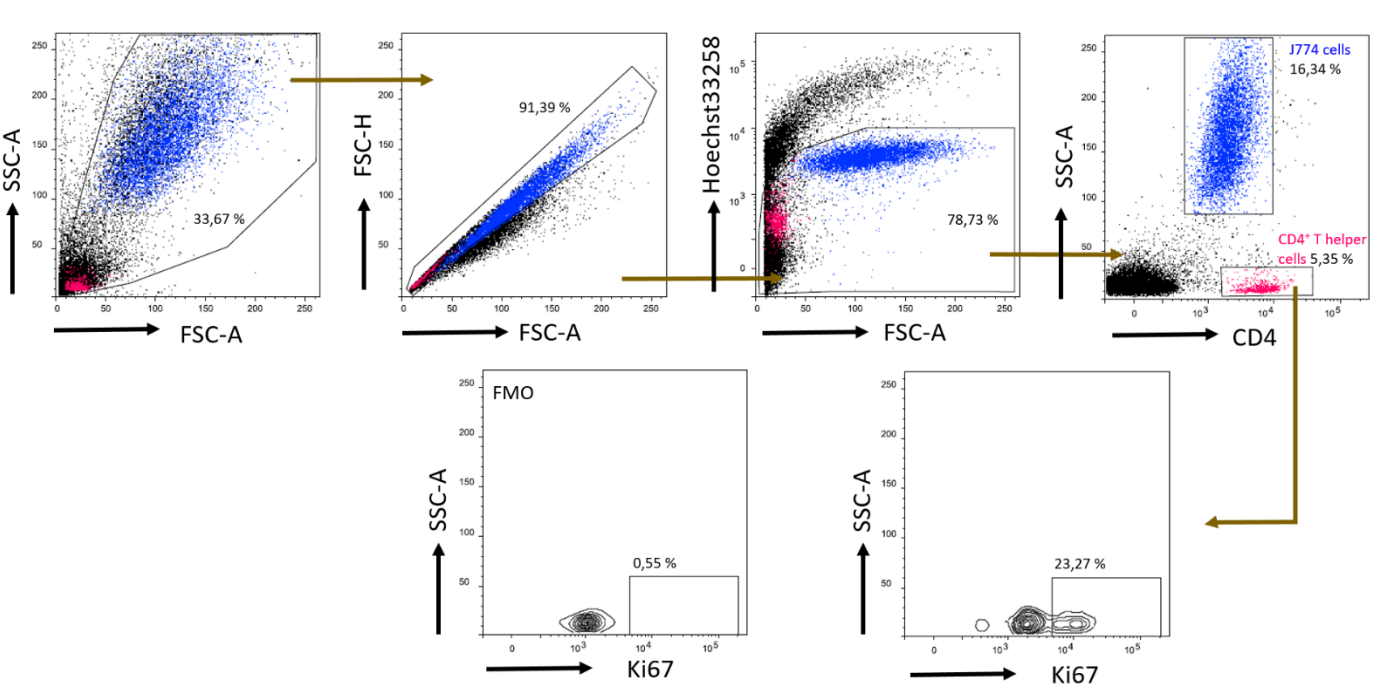


Figure S4 Gating strategy to determine the rate of lymphocyte proliferation. Affected macrophages J774 were co-cultured for 72 hours with sorted CD4 positive cells with capsaicin or/and lipopolysaccharide. Afterwards, presence of intracellular proliferative marker Ki67 was determined by flow cytometry. Gating strategy: debris was excluded based on forward and side scatter (FSC, SSC), then doublets were excluded, and live cells were gated based on Hoechst 33258 negative population (live). T helper lymphocytes were gated based on CD4 positivity and low SSC. The back-gating was used to visualize lymphocytes and macrophages on previous dot plots (pink – $CD4^+$ T helper cells, blue – J774). Proliferated T helper cells were determined by transcriptional factor Ki67. The percentage of proliferating cells ($Ki67^+$) was determined from them.

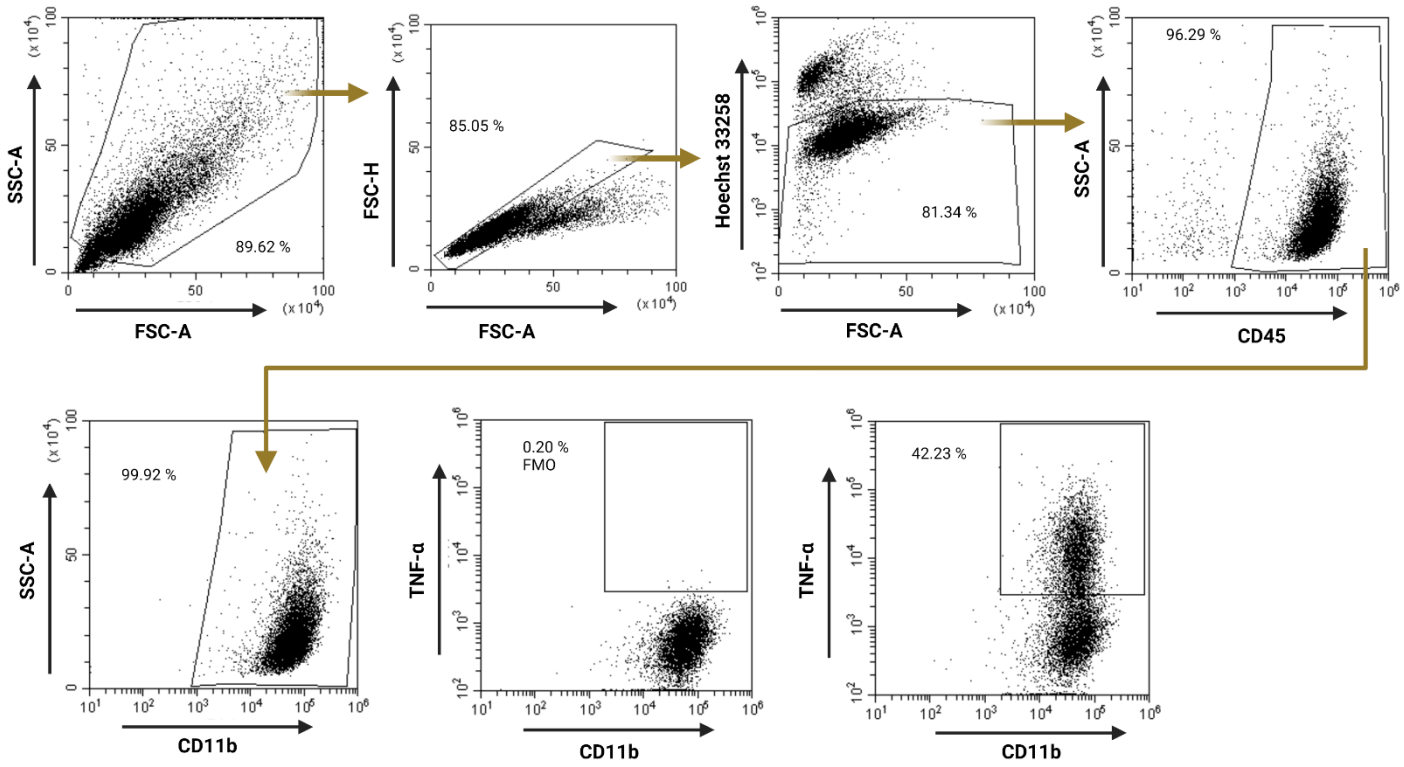


Figure S5 Gating strategy for intracellular cytokines determination. BMDM were preincubated for 4 hours and simulated for 24 hours with capsaicin or/and lipopolysaccharide. Afterwards, expression of surface markers was determined by flow cytometry. Gating strategy: debris was excluded based on forward and side scatter (FSC, SSC), then doublets were excluded, and live cells were gated based on Hoechst 33258 negative population (live). Macrophages were selected based on CD45 and CD11b positivity. From $CD45^{+}CD11b^{+}$ cells were determined percentage of TNFα, IL-1β and IL-6 cells.

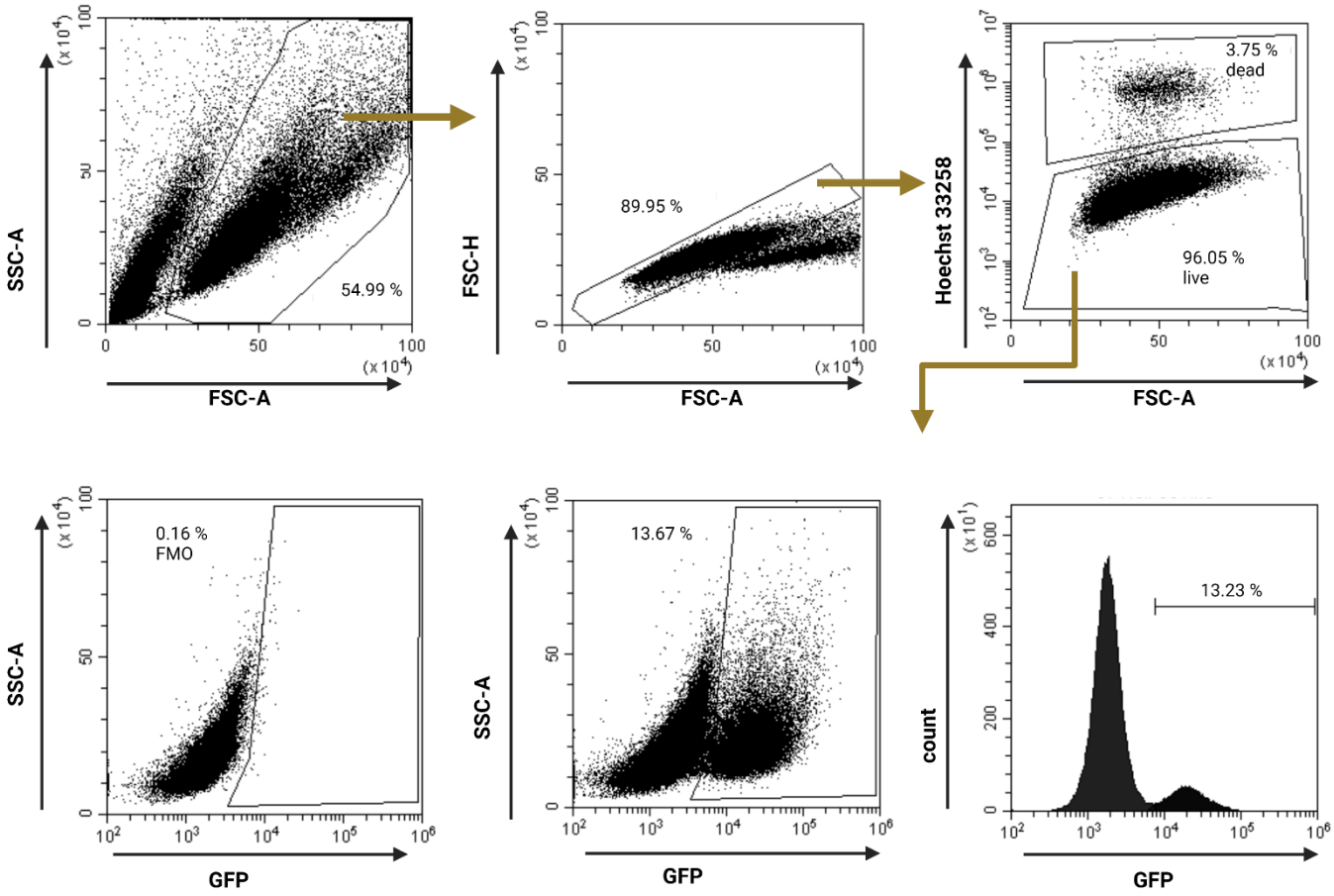


Figure S6 Gating strategy for *Leishmania mexicana* infection. GFP-labelled labelled *L. mexicana* (MNYC/BZ/62/M379) was co-cultured with affected J774 by capsaicin or/and lipopolysaccharide. Amount of phagocytosed parasites was determined by flow cytometry. Gating strategy: debris was excluded based on forward and side scatter (FSC, SSC), then doublets were excluded, and cells were divided into live and dead according to Hoechst33258 positivity. The percentage of cells positive for the GFP signal from the parasites and mean fluorescent intensity was determined.

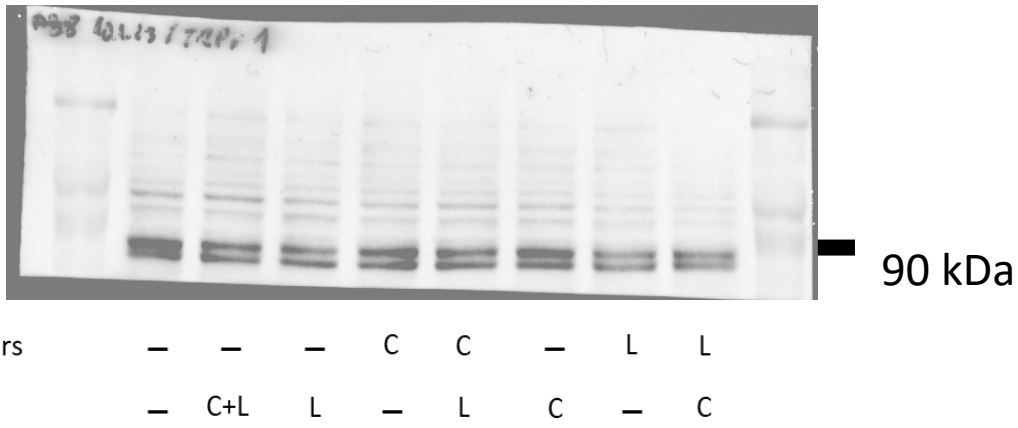


Figure S7 TPRV1 protein expression. Representative Western Blot from J774 cell line preincubated for 4 hours and stimulated for 5 minutes by capsaicin (C) or lipopolysaccharide (LPS).

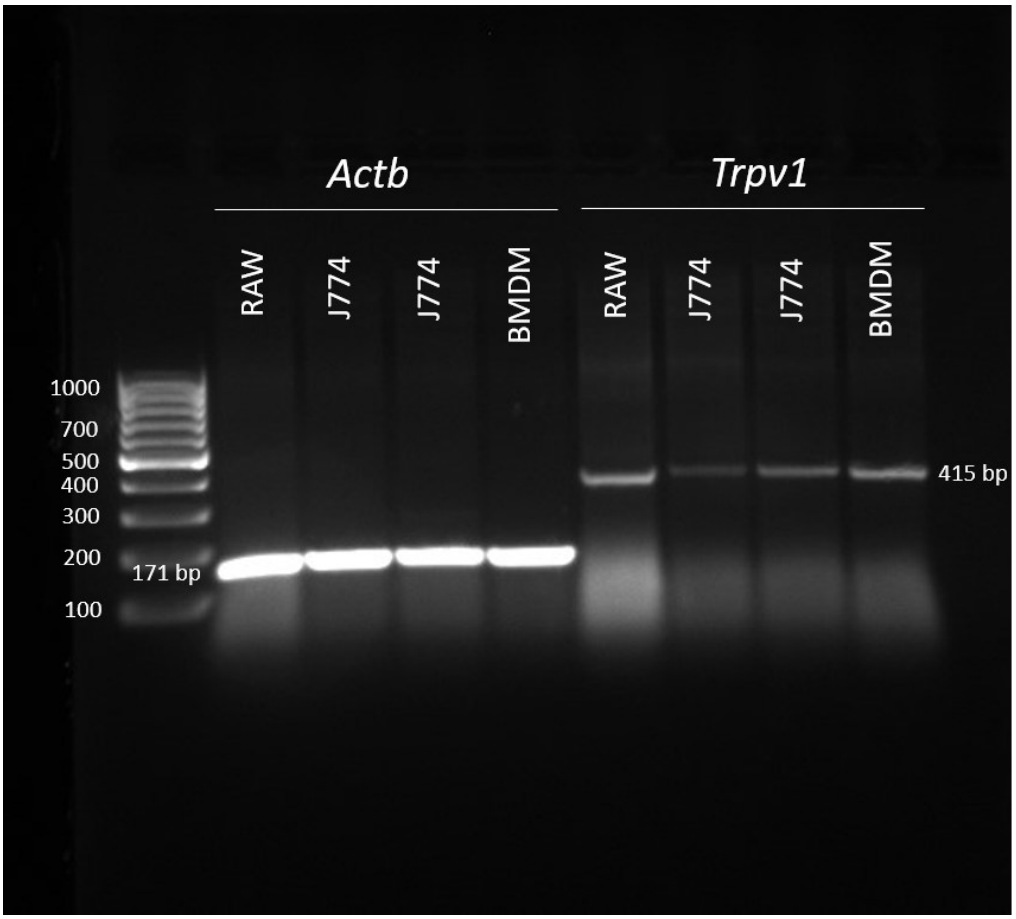


Figure S8 *Trpv1* gene expression. Full-length gel image of *Trpv1* and *Actb* expression. Total RNA was isolated from the J774.2 cell line (J774), bone marrow-derived macrophages (BMDM) and RAW 264.7 cell line (RAW), prepared cDNA and the presence of *Trpv1* gene expression (415 bp fragment) verified by RT-PCR. *Actb* expression (171 bp fragment) was used as a control.

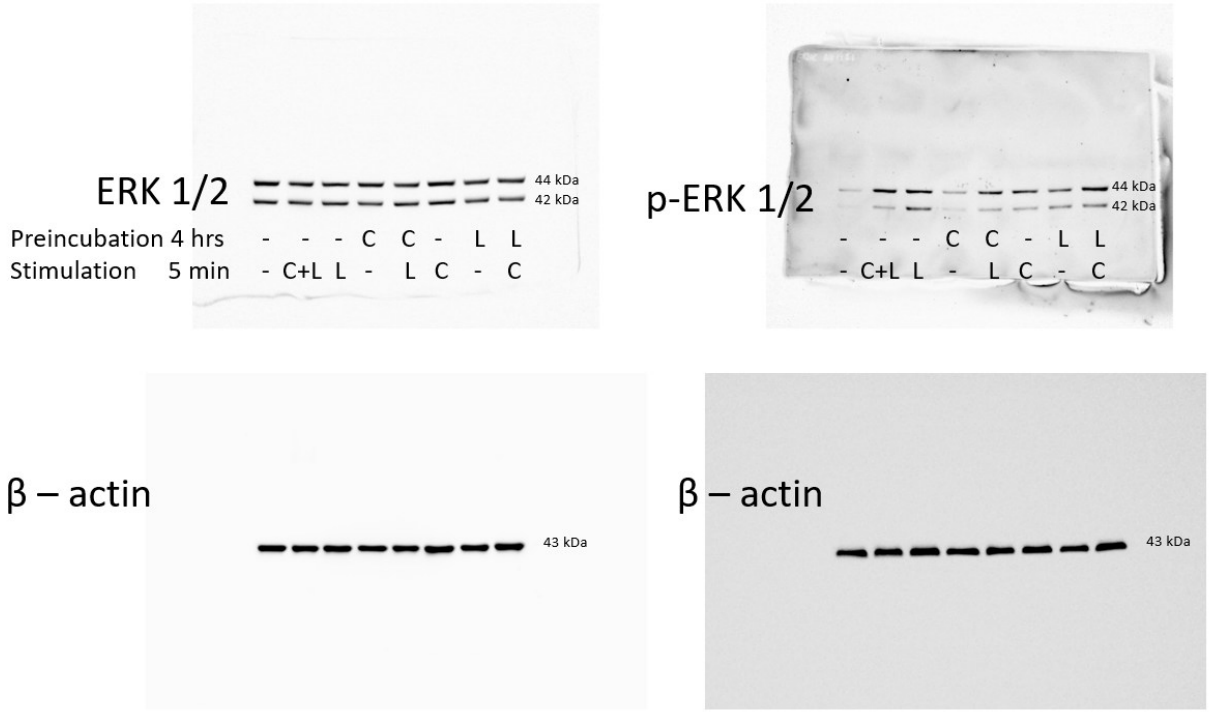


Figure S9 ERK 1/2 protein expression. Representative full-length Western Blot with loading control membranes from J774 cell line preincubated for 4 hours and stimulated with capsaicin (C) or LPS (L) for 5 minutes.

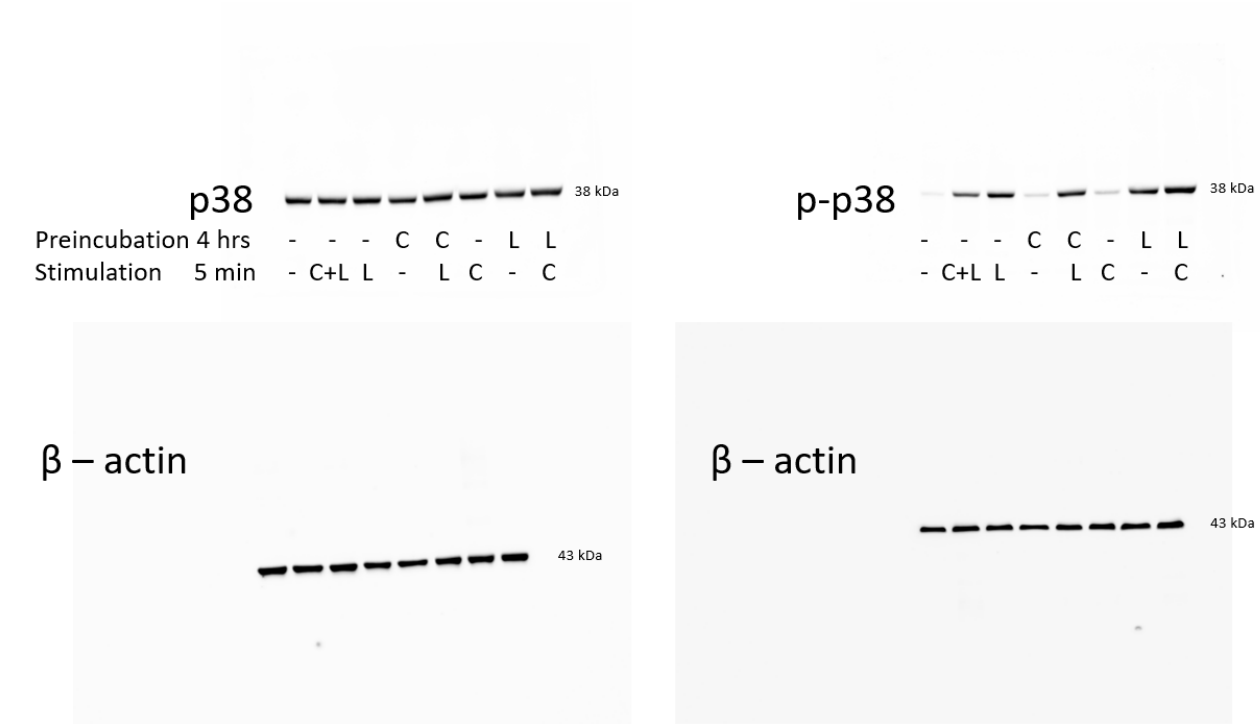


Figure S10 p-38 protein expression. Representative full-length Western Blot with loading control membranes from J774 cell line preincubated for 4 hours and stimulated with capsaicin (C) or LPS (L) for 5 minutes.

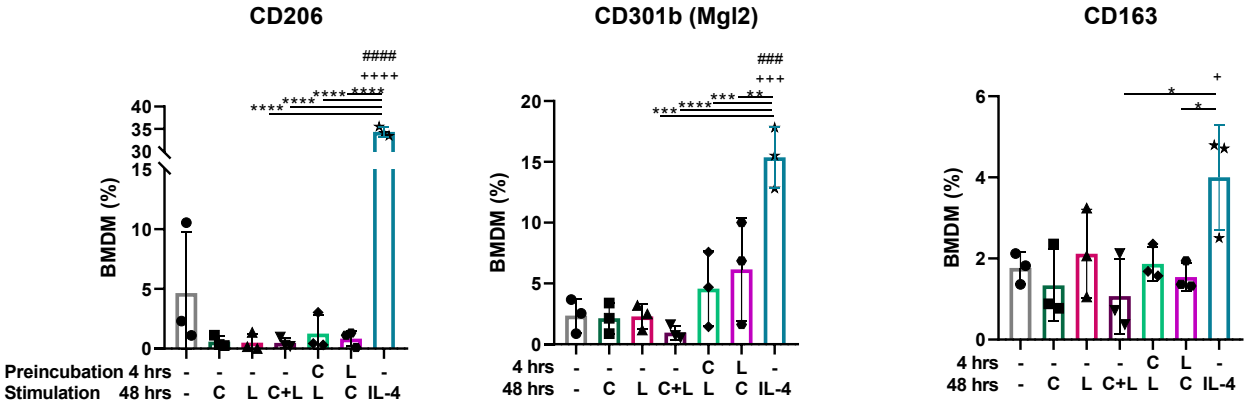


Figure S11 The expression of surface markers and cytokines on BM-derived macrophages. Cells were preincubated for 4 h and cultured with capsaicin (C) or LPS (L) for 48 h. Interleukin 4 (IL-4, 20 ng/ml) was used like positive control. Proportion of BMDM positive for CD206, CD301b (Mgl2) and CD163 was determined by flow cytometry. MFI of CD86 (**a**) or MHCII (**b**) from BM-derived macrophages and the proportion of TNFSF14^{+} (**c**), IL-1β^{+} (**d**), TNFα^{+} (**e**) and IL-6^{+} (**f**) was determined by flow cytometry. Data are shown as means ± SD from three independent experiments. The level of statistical significance was determined using one-way ANOVA followed by Tukey's test for multiple comparisons ($^{n}p < 0.05$, $^{nn}p < 0.01$, $^{nnn}p < 0.001$, $^{nnnn}p < 0.0001$). $^{\#}$ indicates significance from (– –) group. $^{+}$ indicates significance from (– C) group. Other statistical significances between groups are indicated by *.

Discussion I.

The TRPV1 protein has been the focus of the most extensive research within the TRP family, particularly with regard to its function in nociception. Nevertheless, the expression of this protein is not exclusive to neurons, as it has also been detected in immune cells, where its connection to the inflammatory response and pain has been studied [70],[87]. In the attached study, we confirm TRPV1 presence in macrophages at both the RNA and protein level. To assess its expression, we employed a combination of PCR and microscopy, along with Western blot analysis on two different macrophage sources: the murine line J774 and the primary murine BMDM. These findings are in alignment with previously published reports on TRPV1 expression [96],[97]. Since TRPV1 activated by high temperature allows the influx of ions into the cell (and within the cell between individual compartments), thereby triggering various signaling cascades [87],[105], studying its role in inflammation is of great interest. However, TRPV1 activation by elevated temperature poses significant challenges in experimental settings, as elevated temperature in culture has been observed to affect numerous processes beyond TRPV1 itself, including cell growth rate, cell cycle, cell metabolism, and the expression of various other molecules. In the context of immune cells, temperature also impacts their activation status and antigen presentation [87]. Consequently, we opted to utilize its natural ligand, capsaicin. This approach avoids the activation of other adaptive mechanisms in response to elevated temperature, allowing a more focused investigation into the specific effects of the temperature-dependent TRPV1 channel.

As has been noted in several instances throughout the work, the results obtained are sometimes contradictory. The subject of discussion has been the inflammatory or anti-inflammatory role of TRPV1 in immune cells [88],[96],[138],[139],[229],[230]. Under our conditions, as expected, macrophage activation was not observed in response to capsaicin alone. Therefore, an inflammatory environment was established by adding LPS. Induction of inflammation by activating TLR is a well-established experimental procedure. As previously mentioned in the Introduction and the objectives of the work, we hypothesized that the observed variations in results could be attributed to the temporal sequence of experiments and the order of the administration of the stimulation. Therefore, we decided to stimulate macrophages with capsaicin at different time points: before, after, and simultaneously with LPS activation.

The most significant finding of this study was the observation that capsaicin-induced calcium influx was significantly increased in macrophages that were pretreated with LPS. This finding suggests that the TRPV1 channel becomes more sensitive to subsequent stimulation following TLR4 activation. This phenomenon has been observed primarily in neurons, where it has been studied in the context of neuropathic pain [226],[227]. Given the high prevalence of TLR4 in macrophages, it is reasonable to hypothesize that there may be an interaction between TRPV1 and TLR4. Furthermore, we demonstrate the involvement of the ERK1/2 MAP-kinase signaling pathway in the immune response of TRPV1. However, the results indicated that the

phosphorylated form of ERK1/2 did not correspond to the amount translocated to the nucleus. It is postulated that the subcellular localization of activated ERK1/2 plays a pivotal role in various critical cellular processes, such as proliferation and apoptosis [231], yet the effect of its translocation on the immune response remains unclear. This discrepancy could help explain the inconsistencies in the literature regarding the role of ERK phosphorylation in pro- or anti-inflammatory cytokine production [232],[233].

The question remains whether the same effect would be observed if the temperature were increased in macrophages and thus directly activated TRPV1. It is likely that this would result in a similar influx of calcium ions, which could subsequently trigger the phosphorylation of ERK1/2. The activity of the ERK signaling pathway has previously been described in response to heat stress. Increased temperature has been shown to trigger the expression of heat shock proteins (HSP) through regulation of ERK1/2 [234],[235] and to cause down-regulation of tight junction proteins [236]. However, to our knowledge, a direct connection to TRPV1 activity remains to be established. However, based on the findings of our attached study, it can be concluded that TRPV1 could be an important sensor involved in the recognition of heat stress and potentially contribute to the activation of the ERK1/2 pathway. However, it is important to recognize that this hypothesis requires further experimental validation.

In the attached publication, an inflammatory M1 macrophage phenotype is induced by LPS. Subsequent treatment of these cells with capsaicin resulted in a phenotype that closely resembled M2b. However, the obtained macrophages did not fully align with the established characteristics of the M2b phenotype. Consequently, we designated these macrophages as M2b-like. M2b is characterized by antigen presentation and the presence of costimulatory molecules, features we observed in our LPS-stimulated cells prior to TRPV1. In contrast to the M1 phenotype, M2b macrophages exhibit reduced but not completely suppressed pro-inflammatory cytokine production, with a concomitant increase in anti-inflammatory IL-10. Our observations revealed a decrease in TNFα expression at both gene and protein level in LPS-pretreated J774 cells, while IL-1β expression remained largely unchanged. The intracellular levels of TNFα, IL-1β, and IL-6 were significantly decreased in BMDM. However, significant increase in anti-inflammatory cytokines was not observed in neither J774 cells nor BMDM. In line with these observations, other researchers have reported a reduction in inflammatory cytokine production following TRPV1 activation in various *in vitro* models of LPS-induced inflammation [87]. However, to the best of our knowledge, no studies have documented a shift towards high IL-10 production by macrophages in response to this stimulation. Another significant marker of M2b macrophages is TNFSF14 [120]. In the J774 cell line, TNFSF14 was not expressed; however, capsaicin activation significantly increased its expression in BMDM pre-stimulated with LPS. In addition to TNFSF14, other characteristic markers of M2 macrophages, including CD163, CD206, and Mgl-2, were examined; however, no alterations were detected in these markers during the course of the stimulation. The activation of the TRPV1 channel prior to LPS-induced inflammation did not result in the suppression of inflammatory cytokine release, a finding that aligns with the existing literature

[138]. Additionally, TRPV1 activation alone did not exert a substantial influence on the resulting macrophage phenotype. Consequently, we hypothesize that TRPV1 activation modulates pro-inflammatory cytokine production in a manner contingent on the inflammatory milieu.

A notable finding in the attached publication is the observation that the results obtained from J774 macrophages and BMDMs are not always comparable. It is acknowledged that macrophages can exhibit divergent responses to inflammatory stimuli, depending on their origin [237], as evidenced by the presence of distinct surface molecules and the production of cytokines [238],[239]. This discrepancy may help explain the variations observed in the results. Nevertheless, the combination of two distinct types of macrophages offers a more comprehensive understanding of the role of TRPV1 in its function and inflammatory response.

The observed alteration in macrophage phenotype was associated with an increase in ROS levels, NO levels, and mitochondrial membrane potential, as recorded in the LPS-treated group prior to TRPV1 activation. These alterations are likely associated with increased calcium influx and signify an overall increase in macrophage activation. Although NO production is conventionally associated with the M1 phenotype, the M2b macrophage phenotype is also recognized for its NOS enzyme activity [122]. The literature indicates that TRPV1 KO mice exhibit elevated NO production, suggesting that TRPV1 may negatively regulate NO production after LPS stimulation [240]. In addition, it has been established that TRPV1 forms a complex with eNOS, thereby increasing its activity [241], as well as the reciprocal effect of NO, which sensitizes the TRPV1 channel [242]. In addition, Clark et al. investigated the role of TRPV1 in NO release in a mouse model of LPS-induced sepsis and found higher NO production in TRPV1 KO mice, suggesting that TRPV1 negatively regulates NO production upon LPS stimulation [240]. Our data suggest that this phenomenon is indeed dependent on the time point of TRPV1 activation with respect to LPS stimulation, similar to pro-inflammatory cytokines.

The relationship between NO and NOS enzyme activity and elevated temperature and heat stress is a topic that has been extensively studied. Activated macrophages express NOS enzymes at elevated levels and are the predominant NO producers within the body, thus it can be deduced that they play a crucial role in the thermoregulatory response. Furthermore, research has shown that in the brain, glial cells (a type of macrophage) exhibit a suppressed inflammatory response when subjected to both inflammatory and heat shock, compared to glia that were treated with inflammatory stimuli alone. In this process, iNOS expression, NO production, and HSP induction are involved [243]. Furthermore, NO production is markedly elevated in patients with heatstroke, where NO is a key mediator and an integral component of pathophysiological processes, as well as a central factor linking subsequent neurological and cardiovascular abnormalities [244]. The protective role of iNOS has been thoroughly documented in the context of myocardial infarction, where it serves as the primary mediator of cardioprotection induced by heat stress [245]. Additionally, NO plays an important role in

active skin vasodilation during heat stress in humans [246]. However, the role of NO in heat stress signaling is not limited to animal cells. It is a topic that has been widely studied in plants, where it is involved in key processes such as photosynthesis, accumulation of osmolytes, gene expression, protein modification, and oxidative defense [247]. It is a key regulator of the gene expression of transcription factors that lead to plant thermotolerance [248],[249]. The protective effect of NO against heat stress-induced oxidative damage and the active involvement of NO production in the thermoregulatory response have also been described in fungi [250]. This suggests that NO production serves as a critical regulator of temperature resistance across species, where macrophages may play a primary role. Based on the findings of this study and the existing literature, it can be hypothesized that the cooperation between TRPV1 and NOS enzymes in macrophages may play a significant role in the thermoregulatory process at elevated temperatures.

Once more, we can only hypothesize the potential impact of the increase in temperature on the resulting phenotype of LPS-stimulated macrophages. In the rat muscle model of muscle atrophy, macrophages play a crucial role. Heat treatment has been shown to reduce inflammation and overall muscle atrophy. During the inflammatory response and subsequent heat therapy, an increase in the proportion of M2-like macrophages and a decrease in the proportion of M1 macrophages have been observed. Furthermore, the TNFα production was reduced, but the IL-10 production did not change [251]. These outcomes are closely aligned with our observations in the LPS-induced inflammation model and subsequent capsaicin treatment. In a model of bacterial streptococcal infection, heat-treated macrophages exhibited augmented phagocytic activity and elevated bactericidal capacity, accompanied by diminished TNFα and IL-6 production. In this model, iNOS activity was also affected, and surprisingly higher levels of anti-inflammatory IL-10 were measured [252]. These outcomes align to some extent with our own data. However, the observed variations in IL-10 can be reasonably attributed to the recognition of Streptococcus by TLRs other than TLR4. In a parallel study, Foster et al. examined the impact of mild heat stress on murine macrophages, employing a similar experimental setup with elevated temperature treatments before or after LPS stimulation. In their experiments, the immune response of macrophages was examined at 41.5 °C, a temperature sufficient for TRPV1 activation. Their results showed a clear reduction in the production of inflammatory cytokines and suppression of inflammasome activity [253]. This suggests that during inflammatory responses, elevated temperatures can shift the macrophage polarization toward the M2-like phenotype, thereby supporting our observations after activation of the temperature-sensitive channel TRPV1 using capsaicin.

In our study, we aimed to investigate the effect of TRPV1 activation subsequent to LPS stimulation on the macrophage polarization. To achieve this, we employed an infection model utilizing the intracellular parasite *Leishmania mexicana*. It is well established that the phenotype of macrophages exerts a significant influence on the progression of parasitic infections [254]. We hypothesized that the induced M2b-like subtype of macrophages would create a more favorable environment for parasite survival. We supported the hypothesis by

finding a higher percentage of infected macrophages. In the context of elevated temperature, it can be hypothesized that macrophages will exhibit heightened susceptibility to infection by *L. mexicana* in the inflammation model. However, this prompts a further examination of the parasite's own response to elevated temperatures. It is noteworthy that parasites such as Leishmania exhibit a particularly intriguing response to temperature changes, as an increase in temperature constitutes an integral component of their life cycle upon entering the host organism. In various Leishmania species, the synthesis of specific proteins upon exposure to higher temperatures has been confirmed. These proteins may play a role in adaptation to higher temperatures, in parasite cytodifferentiation, as well as in virulence itself [255],[256]. This suggests that the parasite has adapted to temperature fluctuations and should not be fundamentally affected by elevated temperatures. Volpedo et al. have identified a noteworthy correlation between TRPV1 and infection with the parasite *L. mexicana*. In their study of skin lesions caused by *L. mexicana*, they identified macrophages that produce purines with anti-inflammatory and analgesic effects. These purines act through adenosine receptors, inhibit TRPV1 channels, and promote IL-10 production [257]. This finding stands in contrast to the observations recorded in our study, which demonstrated that TRPV1 activation promoted parasite survival and infection. The observed discrepancy may be attributed to the temporal dynamics of TRPV1 channel activation/inhibition, the utilization of an LPS-induced inflammatory model, or the location of TRPV1 channels in sensory nerves compared to immune cells. Nevertheless, it is reasonable to hypothesize that TRPV1 channels in macrophages may also be influenced in a similar manner. Consequently, further research is necessary to elucidate the precise relationship between TRPV1, elevated temperatures, and macrophage responses to *L. mexicana* infection.

II. Immune response to cold exposure: Role of γδ T cells and TLR2-mediated inflammation

In this article, I focus on the immune mechanisms activated after exposure to cold. The mammalian body exhibits remarkable adaptability to cold exposure, involving intricate adjustments in cellular metabolism to facilitate thermogenesis. However, cold-induced stress can influence the immune response, primarily through norepinephrine-mediated pathways. In this study, we used a rat model subjected to short-term or long-term mild cold exposure to investigate the systemic immune response during cold acclimatization. To increase relevance to humans, we validated the data obtained in the rat model in a group of volunteers who regularly bathed in cold water. Our results revealed a complex interplay between cold exposure, neural signaling, immune responses, and thermogenic regulation. One day of cold exposure induced a stress response, including cytokine production in WAT, which subsequently activated BAT and initiated thermogenesis. We also examined the systemic immune response, focusing on leukocyte levels and cytokine production. Notably, γδ T cells emerged as potential regulators of the broader systemic response, suggesting their involvement in the dynamic process of cold adaptation. To explore the mechanisms underlying the role of γδ T cells in this response, we used RNA sequencing. In addition, we challenged cold-exposed rats with a Toll-like receptor 2 agonist and observed a significant modulation of the immune response. This article provides valuable insights into the physiological adaptations that occur in response to cold exposure and highlights the intricate mechanisms governing cold-induced immune and thermogenic responses.

D. Vašek´s contribution on this publication:

In this study, I participated in the design and planning of the experiments. Subsequently, I performed the majority of the experiments. I was also involved in analyzing all the data, which I then visualized. I also participated significantly in the preparation of the manuscript. My overall contribution was approximately 80 %.

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Research Article

Immune response to cold exposure: Role of γδ T cells and TLR2-mediated inflammation

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The mammalian body possesses remarkable adaptability to cold exposure, involving intricate adjustments in cellular metabolism, ultimately leading to thermogenesis. However, cold-induced stress can impact immune response, primarily through noradrenaline-mediated pathways. In our study, we utilized a rat model subjected to short-term or long-term mild cold exposure to investigate systemic immune response during the cold acclimation. To provide human relevance, we included a group of regular cold swimmers in our study. Our research revealed complex relationship between cold exposure, neural signaling, immune response, and thermogenic regulation. One-day cold exposure triggered stress response, including cytokine production in white adipose tissue, subsequently activating brown adipose tissue, and inducing thermogenesis. We further studied systemic immune response, including the proportion of leukocytes and cytokines production. Interestingly, γδ T cells emerged as possible regulators in the broader systemic response, suggesting their possible contribution in the dynamic process of cold adaptation. We employed RNA-seq to gain further insights into the mechanisms by which γδ T cells participate in the response to cold. Additionally, we challenged rats exposed to cold with the Toll-like receptor 2 agonist, showing significant modulation of immune response. These findings significantly contribute to understanding of the physiological acclimation that occur in response to cold exposure.

Keywords: Cold exposure · Cytokines · Immune response · γδ T cells · TLR2

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Cold acclimation is a complex physiological process that involves the activation of brown adipose tissue (BAT) to maintain body temperature. During cold exposure, adaptive thermogenesis occurs, leading to lipolysis, degradation of fatty acids, glucose

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uptake, and the release of norepinephrine (NE). NE, in turn, stimulates brown adipocytes to express and activate uncoupling protein 1 (UCP1), a key regulator of thermogenesis [1]. Elevated levels of UCP1 have been associated with increased glucose uptake and higher energy expenditure, leading to improved whole-body metabolism [2, 3]. Consequently, BAT has emerged as a promising target for the treatment of metabolic disorders and obesity [4, 5].

Recent studies have highlighted the involvement of various immune cell populations in the regulation of BAT activity through complex intercellular networks. The presence of type 2 immune response-associated cells, including type 2 innate lymphoid cells (ILC2) and invariant natural killer T cells, within BAT has been implicated in the modulation of thermogenesis. These immune cells support the functions of eosinophils, regulatory T cells (Tregs), and M2 macrophages, and their crosstalk with brown adipocytes is believed to be critical for metabolic adaptation to cold exposure [6–8]. Additionally, Kohlgruber et al. associated the control of thermogenesis with the homeostatic population of IL-17-producing γδ T cells. IL-17A and TNF-α signaling have been shown to upregulate the expression of thermogenic genes, including *Ucp1*, in brown adipocytes, and induce the production of IL-33 by adipose stromal cells, thereby promoting the action of ILC2 and the maintenance of Tregs. Accordingly, mice deficient in γδ T cells or IL-17 exhibited impaired regulation of adaptive thermogenesis [9]. Moreover, the effect of γδ T cells on thermogenesis has been linked to the IL-17F/IL-17 receptor C pathway, which promotes sympathetic innervation mediated, at least in part, by the production of TGF-β in adipocytes [10].

Although the understanding of immune cell dynamics during the cold exposure is still limited, it is well-established that immune system activation imposes significant energy demands and competes with other physiological processes such as thermoregulation. Recent studies in birds and mice have highlighted the impact of increased energetic demands during cold acclimation on immune responses, leading to enhanced disease tolerance and attenuation of autoimmune conditions [11–13].

Furthermore, investigations into the effects of cold stress on immune function have revealed intriguing findings. Vargovic et al. [14] observed an upregulation of genes associated with anti-inflammatory mediators, markers of M2 macrophages, and eosinophil-associated molecules in the white adipose tissue (WAT) of rats exposed to reduced temperature for 7 days. Conversely, the expression of proinflammatory cytokines and monocyte markers was inhibited in these conditions. In a separate study, Becker et al. [15] observed an increase in the population of Tregs in humanized mice stimulated with β3-adrenergic receptor agonists or in humans after short-term cold exposure. Additionally, Hu et al. [16] found that plasma levels of IFN-γ, IL-2, IL-4, and the percentage of $CD4^+CD25^+Foxp3^+$ Treg cells in the peripheral blood were decreased in rats subjected to short-term cold stress.

Collectively, data from the literature provide evidence that cold stress disrupts the intricate network of signals involved in communication between the nervous, endocrine, and immune systems, leading to impaired immune function. However, it is noteworthy that cold acclimation has been associated with the promotion of an anti-inflammatory environment in various experimental models [16–18].

These findings suggest a complex interplay between cold exposure, thermogenesis, and immune responses, highlighting the need for further research to elucidate the underlying mechanisms. To investigate the interplay between cold exposure, thermogenesis, and immune responses, we conducted a study using a rat model of mild acclimation. The rats were exposed to short-term or long-term cold conditions, and we examined the changes in the immune system. Additionally, we challenged the rats with Pam2CSK4 to evaluate TLR2-mediated immune response. TLR2 is expressed by different types of immune cells, but their expression varies depending on the organism [19, 20]. Furthermore, to validate our findings in a relevant human model, we also included a group of regular cold swimmers in our study.

Results

The weight of BAT is increased after 14 days of cold exposure and cytokine production differs in BAT and WAT

We first aimed to explore the effects of cold exposure on BAT activation. To achieve this, three groups of rats were established: a control group maintained under laboratory temperature conditions, a group exposed to cold for 1 day, and a group exposed to cold for 14 days. Thus, we considered the group exposed to cold for 14 days as cold-adapted. The morphology of BAT is temperature dependent (Fig. 1A). Previously, we have characterized BAT maturation during cold acclimation in the given condition [21]. Additionally, the weight of BAT significantly increased in the group subjected to 14 days of cold exposure, as demonstrated by the data depicted in Fig. 1B. To ascertain whether the observed changes in cytokine production were specific for BAT, we compared cytokine levels in BAT and WAT. Overall, BAT exhibited higher cytokine production than WAT. However, the level of most cytokines (TNF-α, IL-1β, IL-4, IL-6, and IL-10) did not exhibit significant changes in response to cold exposure. In contrast, WAT displayed a notable pattern of cytokine production. After 1 day of cold exposure, there was a significant increase in cytokine levels, followed by a subsequent decrease in the 14-day acclimated group. It is noteworthy that IL-17A and IL-17F were exceptions to this trend, as their levels in BAT from the 14-day cold-acclimated rats showed a significant increase (Fig. 1C).

Cold-acclimated rats displayed an increase in γδ T cells throughout the examined tissues

Subsequently, our investigation focused on evaluating changes in immune cell populations in the spleen and peritoneum of cold-acclimated rats. To identify T cells, we employed the CD3 marker, while the commonly utilized CD45RA marker for the detection of

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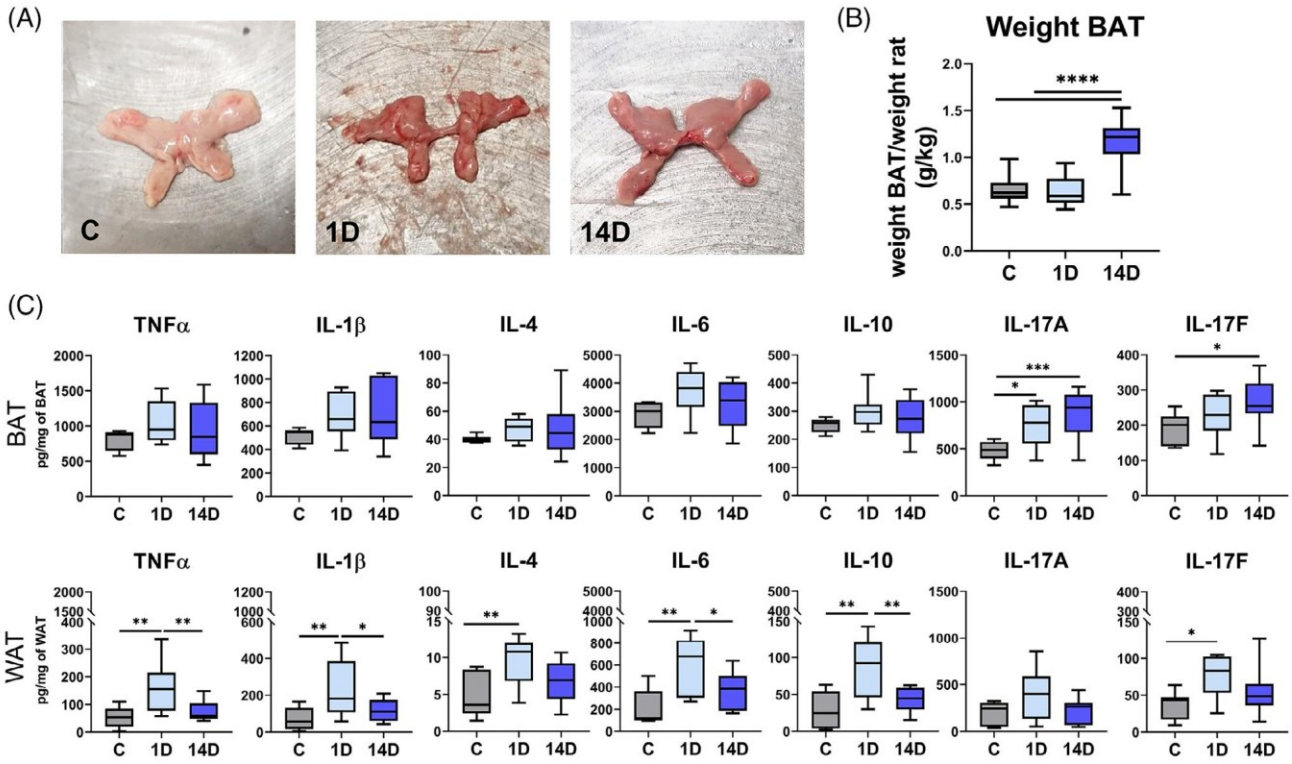


Figure 1. Cytokine production in adipose tissue. (A) The rats were exposed to cold for 1 day (1D) or 14 days (14D) or kept at room temperature (C). Brown adipose tissue (BAT) was isolated from the interscapular area. Representative image. (B) The weight of BAT was normalized to the weight of the respective rat (n = 16). (C) Homogenates were prepared from BATs white adipose tissue (WAT) and the concentration of cytokines TNF-α, IL-1β, IL-4, IL-6, IL-10, IL-17A, and IL-17F were determined by ELISA (n = 7–12). The data are depicted using box-and-whisker plots, where medians are indicated by lines inside the boxes, and whiskers represent the range of values. Statistical significance was assessed using ANOVA followed by Holm-Sidak's test for multiple comparisons ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

B cells in rats was used, myeloid populations were identified using the CD11b/c marker, macrophages using the CD68 marker, and their M2 subtype using CD163. None of these immune cell populations exhibited significant differences between the three experimental groups (Fig. 2A). However, a noteworthy finding emerged regarding the percentage of γδ T cells, which displayed a significant increase in the 14-day acclimated group in all examined tissues. This increase was not limited to the spleen and peritoneal cavity but was also observed in PBMCs and thymus, indicating a systemic induction of γδ T cells due to cold adaptation (Fig. 2B). Even though the highest percentage of γδ T cells was observed in the spleen after 14 days of cold acclimation, γδ T cells isolated from rats exposed to cold for 1 day exhibited increased levels of the proinflammatory cytokines IL-17 and TNF-α, suggesting their activation (Fig. 2C).

Cold-acclimated rats displayed an altered immune response to the TLR2 agonist Pam2CSK4, including NE serum levels, ROS and NO release, and differential cytokine profiles

In light of the observed changes in immune cell populations following cold acclimation, we further investigated the effect of stimulation with infectious agents on the immune system of cold-acclimated rats. Given the significant role played by γδ T cells during cold exposure and considering that *Mycobacterium tuberculosis* infection has been reported to induce γδ T-cell immune responses, we chose to employ Pam2CSK4, to challenge both control and cold-acclimated rats. The weight of BAT did not change after Pam2CSK4 peritoneal injection compared with unchallenged groups. However, there were significant changes in cytokine production in both BAT and WAT (Supporting information Fig. S1). In the peritoneal cavity, TLR2 stimulation led to an increase in the activated $CD80^{+}CD11b/c^{+}$ population (Fig. 3A). In the spleen, we observed an increase in the CD11b/c population, which was accompanied by an elevated intracellular level of TNF-α in CD45-positive immune cells (Fig. 3B). Additional information on other immune cell populations in different tissues can be found in Supporting information Fig. S2. Intriguingly, despite the known association between NE and BAT activation [22], the serum levels of NE did not exhibit significant changes during the process of cold acclimation. However, following the challenge with Pam2CSK4, NE levels in the 14-day acclimated group were found to be significantly higher compared with all other groups. In contrast, rats exposed to 1 day of cold were able to prevent an increase in NE serum levels in response to the Pam2CSK4 challenge (Fig. 3C). To gain further insight into the immune response of cold-acclimated

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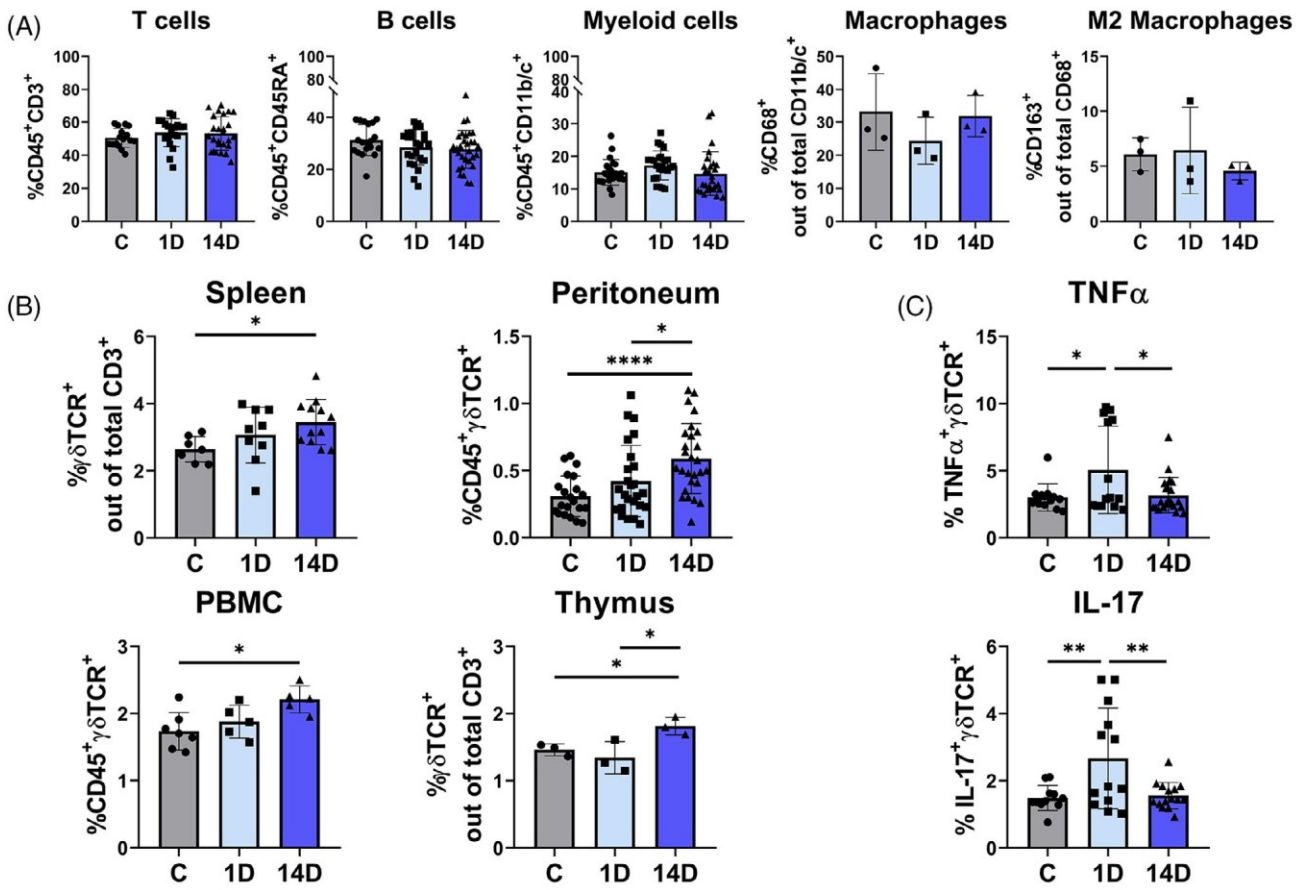


Figure 2. Representation of immune populations. (A) The percentages of T cells ($CD45^+CD3^+$), B cells ($CD45^+CD45RA^+$) myeloid cells ($CD45^+CD11b/c^+$), macrophages ($CD45^+CD11b/c^+CD68^+$), and M2 macrophages ($CD45^+CD11b/c^+CD68^+CD163^+$) were determined from the spleen of rats exposed to cold for 1 (1D) or 14 days (14D), or from the control group (C) using flow cytometry (T cells, B cells, and Myeloid cells $n = 14$; macrophages and M2 macrophages $n = 3$). (B) γδ T cells were evaluated in the spleen ($n = 6$–12), peritoneum ($n = 20$–24), PBMCs ($n = 5$–7), and thymus ($n = 3$) by flow cytometry. (C) Percentages of splenic γδ T cells producing TNF-α and IL-17 were determined by flow cytometry ($n = 9$–14). Data are shown as means with individual values ± SD. Statistical significance was assessed using ANOVA followed by Holm-Sidak's test for multiple comparisons, with significance levels indicated by asterisks ($^*p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$).

rats, we examined the activation of splenocytes from rats both challenged or unchallenged with Pam2CSK4. The results revealed significantly higher production of ROS, NO, and mitochondrial potential in the 1-day cold-exposed group upon TLR2 stimulation. This indicates that short-term exposure to cold may induce a stress response that can influence the immune response to external stimuli (Fig. 3D).

To assess the systemic immune response, we analyzed cytokine levels in the serum using Luminex technology. The levels of cytokines in the serum of control, 1-day exposed, and 14-day acclimated rats did not exhibit significant changes. However, upon TLR2 stimulation, we observed elevated levels of specific cytokines in the challenged rats. Notably, the 14-day acclimated group displayed the highest levels of TNF-α, IL-5, IL-10, and IL-12 (p70). In contrast, rats exposed to 1 day of cold exhibited a significant increase in IFN-γ, IL-2, IL-4, and IL-6. Only the significantly different results obtained from the Luminex array are presented here (Fig. 4).

Bulk RNA sequencing analysis

Since γδ T cells exhibited the most significant changes associated with the cold acclimation, we employed bulk RNA-seq to further investigate mechanisms of their participation in the reaction to the cold. The individual groups were compared with each other (Fig. 5A and Supporting information Fig. S3A) and the obtained data were evaluated by gene set enrichment analysis. In comparison to controls, γδ T cells isolated from the spleen of rats exposed to 1 day of cold exhibited increased gene set related to inorganic cation transport (GO:0019233, e.g. *Cacng6, Cacnb4, Kcnk4, Kcnj2, Scn3b, Scn3a, Nalcn, Trpm8*) and purinergic receptors (*P2rx1, P2ry12)*, but also adenosine A1 receptor (*Adora1*) (Fig. 5B, Supporting information Table S4A and Fig. S4A), chemokines and their receptors (*Cxcl11, Ccl24, Ccl9, Ccl22, Cxcl2, Cxcl13, Cxcr2*), as well as *Il1a* and *Tlr4*. Gene set distinctly increases were also observed for genes involved in lipid catabolic processes (primarily *Cyp39a1, Lipg*, and various phospholipases), as well as various

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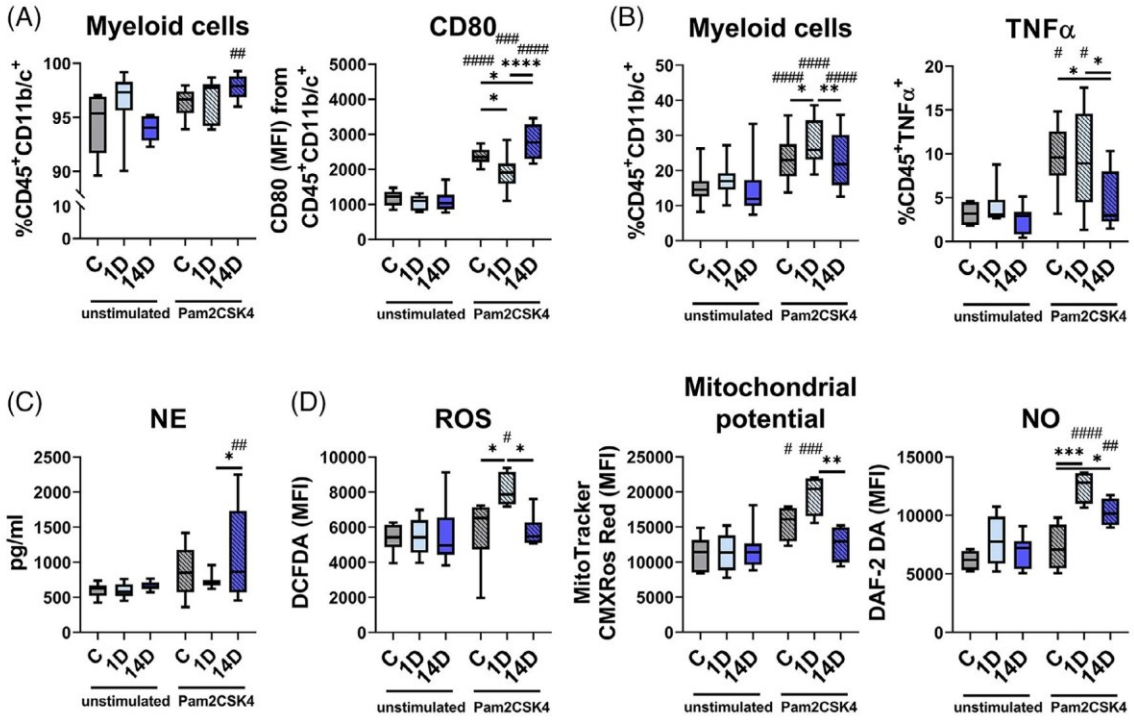


Figure 3. Immune response of cold-exposed rats to TLR2 stimulation. Rats exposed to 1 day (1D) or 14 days (14D) of cold or the controls (C) were challenged with Pam2CSK4. (A) The representation of myeloid cells ($CD45^+CD11b/c^+$) in the peritoneum and their activation rate ($CD80^+$) was determined by flow cytometry (n = 5–9). (B) The representation of myeloid cells ($CD45^+CD11b/c^+$), γδ T cells ($CD45^+CD3^+\gamma\delta TCR^+$), and percentage of $CD45^+TNF\text{-}\alpha^+$ cells from the spleen was determined by flow cytometry (n = 5–9). (C) Norepinephrine concentration was determined by ELISA. (D) Mean fluorescence intensity (MFI) of DCFDA for the presence of ROS, DAF-2 DA for NO, and MitoTracker Red CMXRos for mitochondrial membrane potential were determined by flow cytometry (n = 6–7). The data are depicted using box-and-whisker plots, where medians are indicated by lines inside the boxes, and whiskers represent the range of values. Statistical significance was assessed using ANOVA followed by Holm-Sidak's test for multiple comparisons ($^{x}p < 0.05$, $^{xx}p < 0.01$, $^{xxx}p < 0.001$, $^{xxxx}p < 0.0001$); * indicates significance within the same group. # indicates significance between groups.

genes associated with oxidative phosphorylation (several subunits for NADH dehydrogenases, cytochrome c oxidases, and ATPases). In contrast, the expression of gene sets associated with cellular DNA replication and the cell cycle (rno04110) was downregulated (Fig. 5C, Supporting information Table S4A and Fig. S4A). The reduced cell cycle was confirmed by gene expression of *Mcm2* and *Mki67* determined by qPCR, where the amount of Ki67 was also determined at the protein level by flow cytometry (Supporting information Fig. S3B, D). These changes suggest that γδ T cells exposed to cold-induced stress experience heightened sensory perception, and undergo activation, but simultaneously decrease their division.

When comparing γδ T cells isolated from the spleen of 14 days-cold-acclimated rats with the control group, an increased gene expression was noted in the gene set linked to second-messenger mediated signaling, notably including adrenergic receptors. Moreover, there was an increase in the expression of a gene set associated with antigen processing, presentation, and migration (specifically RT1 class I and II genes), alongside an upregulation of semaphorins (*Sema3e, Sema6b, Sema4c, and Plxna4*) and gene expression of sphingosine-1-phosphate receptor 3 (*S1pr3*). Using qPCR, we confirmed changes in genes associated with the chemotaxis of γδ T cells. In particular, an increase in the expression of Ccr6 was observed in γδ T cells, while the expression of Ccl20 decreased in the spleen (Supporting information Fig. S3C). Downregulation of gene set related to Cytochrome P450 was detected, while genes encoding glutamate receptors (*Grm1, Grm2, Grm3, Grik4, Gria1, Grin2d*) and acetylcholine receptors (*Chrna9, Chrna4, Chrm1*) displayed increased expression (Supporting information Table S4B and Fig. S4B) This suggests that γδ T cells from rats exposed to cold for 14 days respond to distinct neurotransmitter signals compared with rats acclimated for 1 day.

Surprisingly, expression of gene set associated with IL-17 signaling pathway (rno04657) was decreased, including genes form *Il17a* and *Il17f, Il6, Il1b* and *Ifng* and some chemokines (*Ccl12, Cxcl3, Ccl17, Cxcl1, Cxcl10*) (Fig. 5D, Supporting information Table S4B and Fig. S4B). Decreased gene expressions of *Il17a*, *Il17f*, and *Ifng* were also confirmed by qPCR (Supporting information Fig. S3D).

The comparison of samples taken from rats subjected to cold exposure for both 1 and 14 days demonstrated that the gene expression induced by 1-day exposure resembled the gene expression patterns of the controls in the 14-day acclimated group (e.g. re-upregulation of a gene set associated with the cell cycle, Supporting information Table S4C and Fig. S4C) This finding implies that the gene expression changes observed after 1-day exposure

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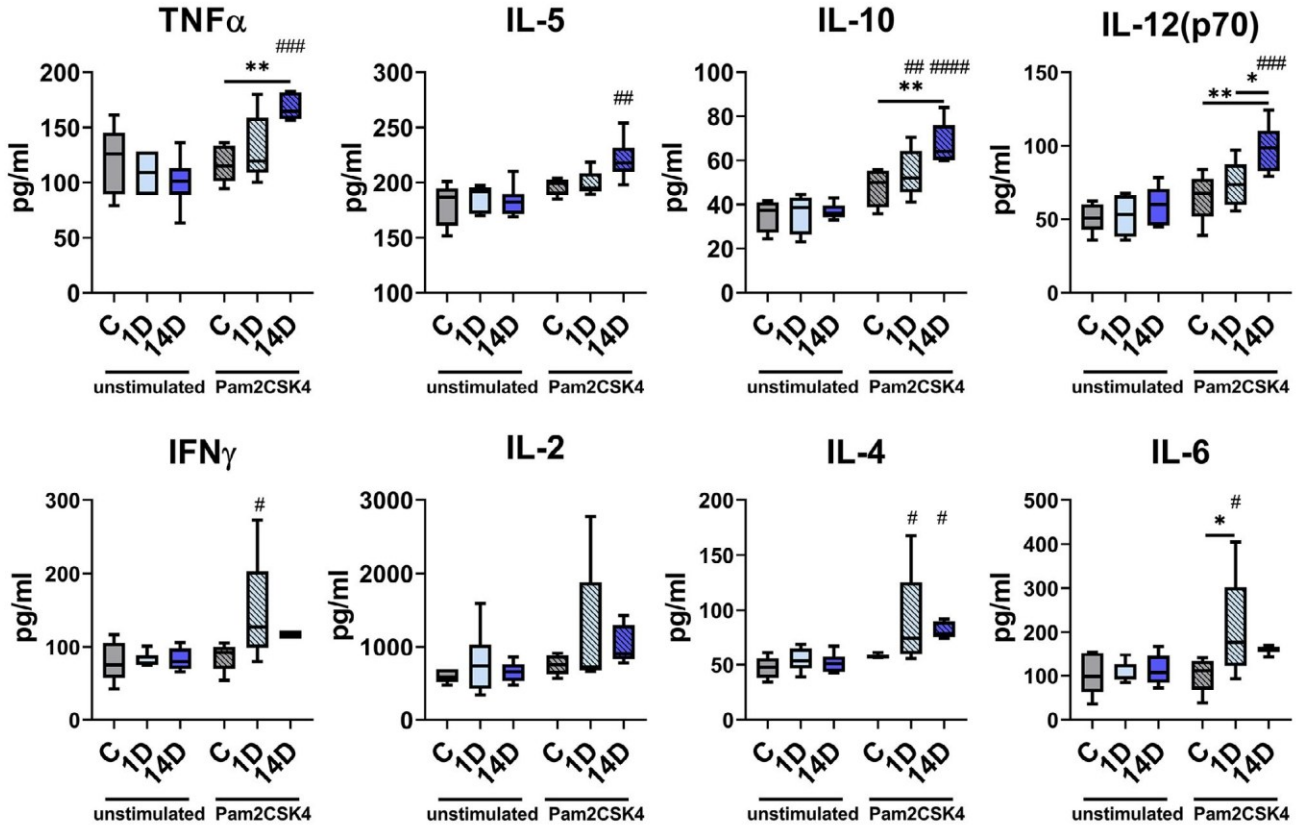


Figure 4. Serum cytokine concentration. Rats exposed to 1 day (1D) or 14 days (14D) of cold or the controls (C) were challenged with Pam2CSK4. The cytokine concentration of TNF-α, IL-5, IL-10, IL-12(p70), INF-γ, IL-2, IL-4, and IL-6 in serum was determined by Luminex. The data are depicted using box-and-whisker plots, where medians are indicated by lines inside the boxes, and whiskers represent the range of values. Statistical significance was assessed using ANOVA followed by Holm-Sidak's test for multiple comparisons $n = 5–6$ ($^{x}p < 0.05$, $^{xx}p < 0.01$, $^{xxx}p < 0.001$, $^{xxxx}p < 0.0001$) * indicates significance within the same group. # indicates significance between groups.

might be transient, suggesting a potential adaptive process over time. Interestingly, when compared with both the control and 1-day groups, gene sets that exhibited elevated expression in γδ T cells obtained from the 14-day adapted rats, were associated with functions like chemotaxis and the regulation of cell adhesion. Notably, these genes included semaphorins, sphingosine-1-phosphate receptors, integrins (such as *Itga5* and *Itgbl1*), and CD209 (Supporting information Table S4C and Fig. S4C). These findings suggest that the gene expression changes observed in γδ T cells during 14 days of acclimation are linked to processes involving cell movement and adhesion modulation.

Regular cold swimmers displayed increased populations of γδ T cells and distinct cytokine response upon stimulation with LPS

To validate the findings observed in the experimental model, we recruited a group of regular cold swimmers and compared their PBMCs to a nonacclimated control group. The populations of $CD45^+CD3^+CD8^+$ ($CD8^+$ T cells), $CD45^+CD3^+CD4^+$ ($CD4^+$ cells), $CD45^+CD3^-CD20^+$ (B cells), $CD45^+CD3^-CD56^+$ (NK cells), $CD45^+CD11b^+CD33^+CD14^+$ (monocytes), and $CD45^+CD11b^{-/dull}CD11c^+$ (dendritic cells) did not show significant differences between the two groups (Fig. 6A). However, the population of γδ T cells, gated from both total $CD45^+$ leukocytes and $CD45^+CD3^+$ cells, exhibited a significant increase in regular cold swimmers. Further analysis of γδ T cells based on the expression of transcription factors revealed a significant increase in both RORγt and T-bet positive γδ T cells in cold swimmers (Fig. 6B).

To assess the immune response of cold swimmers to infectious agents, PBMCs isolated from both cold swimmers and controls were stimulated with Pam2CSK4. Cytokine analysis showed decreased production, but no significant changes (Supporting information Fig. S5), therefore we decided on LPS stimulation. Interestingly, after LPS stimulation our results demonstrated that cold swimmers exhibited decreased levels of TNF-α and IL-10 compared with the control group, while the production of IL-6 and IL-1β was comparable between the two groups (Fig. 6C).

Discussion

The mammalian body possesses a remarkable ability to adapt to both internal and external changes, such as environmental tem-

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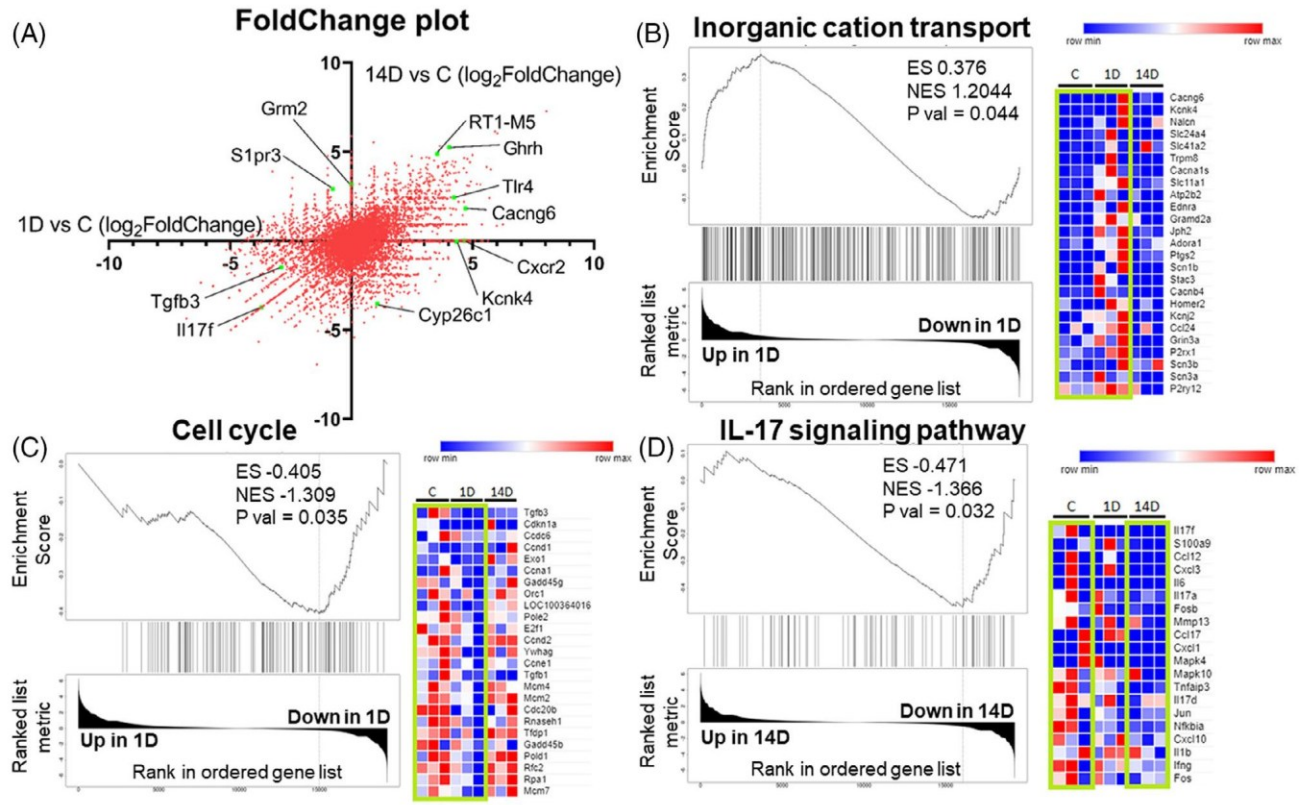


Figure 5. Gene expression analysis of γδ T cells. (A–D) Bulk RNA-sequencing analysis of FACS-sorted splenic γδ T cells isolated from control (C), 1 day (1D) or 14 days (14D) cold-exposed rats. (A) DESeq2 calculated $\log_2$ fold changes for 1D vs. C and 14D vs. C were used for the foldchange plot. (B–D) DESeq2 calculated $\log_2$ fold changes were used as input and analyzed for enrichment of pathways using GO and rno resource. (B) Gene-set enrichment plot comparing 1D and C group for set Inorganic cation transport GO:0019233 (left) and heatmap of the 25 selected important genes from gene set, showing normalized counts reads of individual samples (right). (C) Gene-set enrichment plot comparing 1D and C group for set cell cycle rno04110 (left) and heatmap of the 25 selected important genes from gene set, showing normalized counts reads of individual samples (right). (D) Gene-set enrichment plot comparing 14D and C group for set IL-17 signaling pathway rno04657 (left) and heatmap of the 20 selected important genes from gene set, showing normalized counts reads of individual samples (right).

perature. Cold exposure triggers sympathetic activation and under the influence of beta-adrenergic signaling, brown adipose tissue is formed, and thermogenesis occurs [23]. However, cold-induced stress can also impact immune response in an NE-mediated manner [24].

BAT has been recognized as a highly metabolically active tissue, with various cell types producing substantial amounts of cytokines [25]. In our experimental setup, we opted for the outbred Wistar rat strain, known for its genetic diversity, aiming to achieve results that are more broadly applicable, even though the potential cost of reduced reproducibility. As a result, we can assert that the significant results we obtained have high biological relevance. In this model, subjecting the rats to 14 days of moderate cold acclimation resulted in increased BAT size, yet this was not accompanied by higher relative cytokine production. In contrast, a 1-day cold exposure led to a significant increase in cytokine production in WAT (Fig. 1C). These findings suggest that the initial stress response triggered by cold exposure first mediates changes in WAT associated with cytokine production. These changes then induce subsequent responses leading to the activation of BAT. This is in accordance with Liu et al. [26] who suggested that mature adipocytes coordinate the tissue remodeling, including cell network upon cold exposure. Notably, the exception was the production of IL-17 by BAT, where both isoforms IL-17A and IL-17F increased in cold-exposed animals, with higher production in the 14-day acclimated rats. This finding confirmed the importance of IL-17 signaling in thermogenesis [9, 10]. Important mechanisms may include the metabolic activity of IL-17, which is involved in several processes in adipocytes. These include decreased insulin-mediated glucose uptake as well as increased expression of thermogenic genes associated with increased metabolic respiration [9, 27]. Production of IL-17 has been linked to γδ T cells, which, through IL-17A production, trigger further signaling, including thermogenesis [28]. Overall, it appears that cytokine production by WAT is more stress-related and may be involved in activating the thermogenic function of γδ T cells within BAT. In contrast, BAT exhibits a cold-induced activation associated with sustained cytokine production in cold-acclimated animals. However, the crucial role of γδ T cells in cold acclimation extends beyond adipose tissue, as after 14 days of cold acclimation, the only population that increased was γδ T cells, and higher levels of them were found in the spleen, peritoneal cavity, peripheral blood, and

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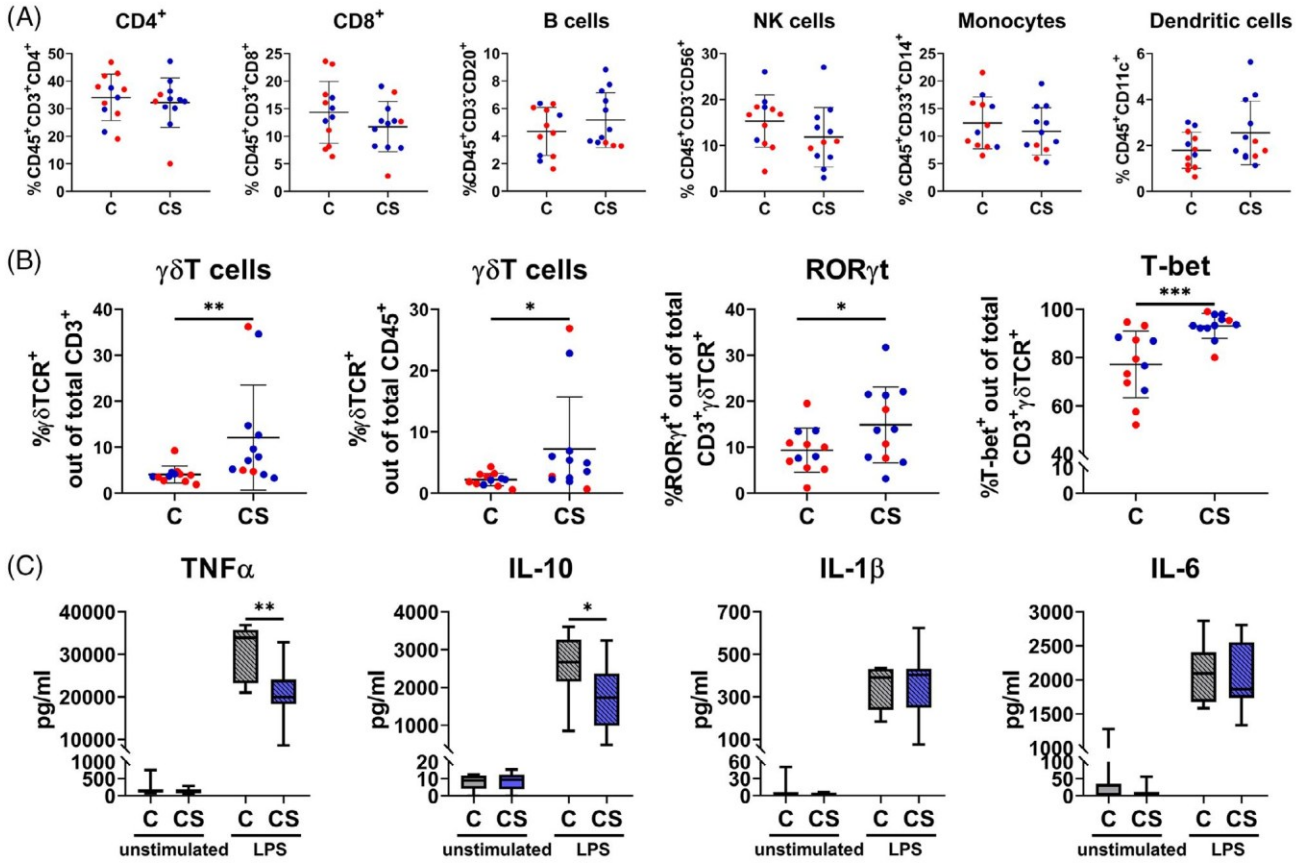


Figure 6. The immune population in regular cold swimmers. (A–C) PBMCs were isolated from human volunteers who regularly swim in cold water (cold swimmers [CS]) and healthy controls (C). (A) The representation of immune populations $CD4^+$ T cells ($CD45^+CD3^+CD4^+$), $CD8^+$ T cells ($CD45^+CD3^+CD8^+$), B cells ($CD45^+CD3^-CD20^+$), NK cells ($CD45^+CD3^-CD56^+$), monocytes ($CD45^+CD11b^+CD33^+CD14^+$), and dendritic cells ($CD45^+CD11b^{-/dull}CD11c^+$) were determined by flow cytometry. (B) The representation of γδ T cells ($\gamma\delta TCR^+$ out of $CD45^+CD3^+$ and $\gamma\delta TCR^+$ out of $CD45^+$) and their transcription factor RORγt and T-bet was determined by flow cytometry. (C) PBMCs were cultivated w/o LPS for 24 h and concentration of cytokines TNF-α, IL-10, IL-1β, and IL-6 was determined by ELISA. (A, B) The data are depicted using individual values (blue – men; red – women) with mean and SD. (C) The data are depicted using box-and-whisker plots, where medians are indicated by lines inside the boxes, and whiskers represent the range of values. (A–C) The data were tested by the Mann–Whitney test or unpaired *t*-test, since it passed the Shapiro–Wilk normality test. $n = 12$ ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

thymus (Fig. 2B). This increased representation indicates an overall higher production of γδ T cells at the systemic level.

Stress has been recognized as a risk factor for various infections [24]. Moreover, it has been demonstrated that TLR4 activation may potentially compromise adaptive thermogenesis [29]. Additionally, mice deficient in TLR2 or TLR4 have exhibited altered thermogenesis [30], prompting us to investigate the TLR2-mediated immune response in our model. The inflammatory response triggered by Pam2CSK4 application involves alterations in neuropeptides, cytokines, hormones, and other factors in the body, which collectively influence all immune cells [31], including γδ T lymphocytes [32]. Upon studying the immune response in the spleen, we observed that immune response to the Pam2CSK4 challenge was mediated by CD11b/c cells and was associated with increased levels of TNF-α. Additionally, we noted significant modifications in the release of NE, a key factor regulating thermogenesis. Both innate and adaptive immune cells express β2 adrenergic receptors (ARs), and β2-AR signaling plays a crucial role in maintaining immune homeostasis [33]. Indeed, the increased markers of activation in splenocytes, including the production of ROS, NO, and mitochondrial potential in the 1-day cold-exposed group upon TLR2 stimulation (Fig. 3D), indicate that cold exposure modifies immune response to pathogens.

Alterations in serum cytokine levels following cold exposure have been observed across various models and contexts [24, 34–36]. Human studies, often involving physical activity and exposure to extremely low temperatures [37–39], contribute to the variable findings in cold-induced cytokine changes. Notably, more intense cold exposure tends to induce more pronounced alterations. In our mild acclimation model, cold exposure alone did not trigger significant cytokine changes, consistent with the findings of Krueger et al. [37]. However, TLR2 stimulation during acclimation, whether short- or long-term, led to increased serum cytokine levels. Interestingly, these changes did not demonstrate clear pro- or anti-inflammatory patterns (Fig. 4). Cold exposure has been shown to engage TLR signaling pathways in fish [36], and another study showed that in vitro cold exposure altered cytokine production in monocytes exposed to LPS [40]. Given that

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cold acclimation activates diverse immune and somatic cell populations at varying levels, stimulating different metabolic pathways [14, 41, 42], these factors likely contribute to the distinct pattern of cytokine production observed. In our model, we suggest that the increased level of circulating cytokines in cold-exposed animals is due to the production of various factors, including neurotransmitters that modulate the sensitivity of TLR activation after cold exposure. Our findings indicate that cells of the myeloid lineage, which are capable of producing a diverse array of cytokines and exhibited alterations in expression following Pam2CSK4 stimulation, may be responsible for the observed change.

We conducted a detailed investigation of γδ T cells using bulk RNA sequencing. Generally, when cells experience distress, they release ATP into the extracellular space, initiating signaling events such as increased intracellular calcium, phospholipase activation, and immune cell activation [43]. Stress-activated adrenergic receptors require purinergic receptors for downstream signaling [44, 45]. Our findings revealed an augmented expression of genes associated with cation channels in γδ T cells, underscoring their pivotal role in cold acclimation, a response that is conserved across species [46]. However, ATP can either inhibit or stimulate lymphoid cell proliferation based on specific cellular subsets and extracellular ATP concentration [47]. Our results suggest that 1-day cold exposure is associated with an activated phenotype of γδ T cells [48, 49], a conclusion supported by the increased levels of TNF-α and IL-17 detected via flow cytometry (Fig. 2C).

During acclimation to cold, there is a shift in neurotransmitter signaling from acute ATP release to a complex interplay between various neurotransmitters. Our results showed increased expression of genes related to the glutamate and acetylcholine response in γδ T cells from cold-acclimated animals. While specific information about γδ T cells is limited, it is known that different T-cell subsets express various types of glutamate and cholinergic receptors, influencing critical functions like adhesion, migration, proliferation, and cytokine production [50, 51]. Additionally, glutamate has a protective role in preventing apoptotic cell death in activated T cells [50], which may be related to the increased proportion of γδ T cells after cold acclimation. This suggests that changes in neurotransmitter levels during cold acclimation closely mirror the regulation of their respective receptor gene expression. We also observed increased gene expression of the semaphorin family in both acclimated groups. These molecules play a role in different phases of the immune response, including migration, proliferation, and activation [52]. In addition, semaphorins have been described as molecules that link the sympathetic nervous and immune systems and have been documented in BAT [53]. This complex interplay likely plays a significant role in shaping the overall immune response and thermoregulation during cold acclimation.

The decrease in genes and proteins associated with IL-17 signaling aligns with the broader anti-inflammatory response induced by cold acclimation [21, 54]. Additionally, the upregulation of genes linked to antigen processing, presentation, and cell migration (Supporting information Fig. S4) indicates enhanced immune responsiveness. Although limited literature exists on these changes, studies have demonstrated increased MHCII antigen presentation activity in adipocytes [26] and heightened migratory capacity of endothelial cells following acute cold stress [55]. The observed increase in the percentage of γδ T cells in the spleen, coupled with enhanced migratory activity, suggests that this acclimation could serve to strengthen the immune system's ability to respond effectively during cold exposure. Spiljar et al. [12] have detected significant changes in genes important for myeloid cells in mice exposed to cold for 2 weeks. Specifically, Ly6C high-positive monocytes showed reduced MHCII expression [12]. The effect of cold on NK cells and their activity is also suggested; however, published results are contradictory [56–58]. This indicates that various immune populations can be differently affected by changes triggered by cold exposure.

The benefits of cold swimming on the immune system are generally acknowledged. However, research on the effects of regular cold immersion has yielded mixed results. This is primarily due to significant variations in participant groups and study protocols. Studies that included experienced cold swimmers showed variations in markers considered, some studies also included exercise, as extensively reviewed by Knechtle et al. and Tipton et al. [59, 60]. Among all the examined immune cell populations, only γδ T cells exhibited significant changes in PBMCs of regular cold swimmers within our group. To our knowledge, γδ T cells have not been studied in this context before. The population of IL-17A-producing γδ T cells has been associated with cold acclimation in animal models [9, 28]. Interestingly, we observed both RORγt and T-bet positive γδ T cells in cold swimmers (Fig. 6B). In a study by Dugué and Leppänen, habitual winter swimmers showed decreased LPS-induced release of IL-1β and IL-6 [61]. LPS is recognized via TLR4 on antigen-presenting cells; however, stimulation of γδ T cells with LPS increased their proliferation and cytotoxic potential [62]. In our group, the levels of the cytokines IL-1β and IL-6 after PBMC stimulation with LPS or Pam2CSK4 did not show significant differences between controls and cold swimmers. However, the levels of TNF-α and IL-10 were decreased in this setting. Cold acclimation challenges both the neuroendocrine and immune systems and strongly depends on the specific acclimation protocol and individual factors. Nevertheless, the results indicate that adaptive mechanisms occur in habitual cold swimmers, which can have varying effects on different immune-cell populations and cytokine responses.

Conclusion

In conclusion, we observed a significant shift in the immune system following the cold acclimation. These changes included alterations in cytokine production in both WAT and BAT. Remarkably systemic shifts occurred in immune populations within the spleen, the peritoneal cavity, and peripheral blood. Prior studies have already established the crucial involvement of γδ T cells present in adipose tissue for thermogenic regulation [9, 10, 25],

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and the results presented in our study further emphasize the systemic role of this cell population. Furthermore, our analysis of the impact of cold acclimation on γδ T cells specifically revealed that under physiological conditions, such as cold exposure, the most significant changes in gene expression respond to neural signals. This indicates that the neuro-immune axis plays a prominent role in this process. Additionally, we have recognized the role of semaphorins, a product of γδ T cells, crucially involved in processes such as migration, proliferation, and antigen presentation. Notably, when rats were challenged with the pathogenic stimulus, the BAT weight remained unaffected, demonstrating the body's priority of maintaining core temperature. However, the immune response to TLR2 stimulation in cold-exposed animals was altered, reflecting a deviation from the response of animals housed in laboratory temperature. This altered immune response was primarily based on cells of the myeloid lineage. Our findings thus shed light on the dynamic regulation of the intricate process of cold acclimation, involving the modulation of immune cells and immune-related products. It is highly plausible to suggest that γδ T cells play one of the important roles in the dynamic regulation of cold acclimatation.

Methods

Cold acclimation protocol

The cold acclimation was performed as previously described [63]. Male Wistar rats (7 weeks old, 200 g body weight, Velaz) were housed in pairs in well-bedded cages to minimize environmental and social stress. All experiments were performed in the "winter-spring" season (from October to March). Rats divided into two experimental groups were acclimated to 8 ± 1°C for 1 day or 14 days. The control group was kept at 24 ± 1°C. The temperature of acclimation was set up just above the threshold of shivering and nonshivering thermogenesis in rats [64]. For TLRs' stimulation, Pam2CSK4, a TLR 2/6 agonist, was used (InvivoGen) dissolved in PBS in a dose of 100 μg/kg injected intraperitoneally for 24 h before the end of the experiment (PBS was injected as a control). At the end of the cold exposure, all animals were anesthetized (thiopental, 60 mg/kg; i.p.) at the respective temperature to avoid an acute thermoregulatory response. The experimental protocol was approved by the Animal Care and Use Committee of the Faculty of Science, Charles University and Ministry of Education, Youth and Sport, Prague, Czech Republic, No. MSMT-31167/2021-4.

Isolation of cells — PBMCs, peritoneum, spleen, and thymus

After the rat was sacrificed, blood was collected in EDTA-coated tubes. PBMCs were isolated by Ficoll-Paque PLUS (GE Healthcare) gradient centrifugation. Peritoneal lavage cells were flushed with Dulbecco's Modified Eagle's Medium from the peritoneal cavity. The spleen and thymus were dissected with scissors and homogenized by microtube plastic pestle homogenizer, and a single-cell suspension was prepared. Red blood cells from the peritoneum and spleen were removed using ammonium-chloride-potassium lysing buffer, and the resulting cell suspensions were analyzed by flow cytometry.

Preparation of adipose homogenate

Inguinal WAT and BAT from the interscapular area were isolated, sectioned, and frozen in PBS. The tissue was sectioned and frozen in PBS. After two freeze-thaw cycles, the homogenates were centrifuged for 5 min at 5000 × *g*. The supernatant was filtered and used for analysis. The concentration of cytokines TNF-α, IL-1β, IL-4, IL-6, IL-10, IL-17A, and IL-17F was measured by ELISA according to the manufacturer's instructions (R&D Systems).

Flow cytometry

Single-cell suspensions were prepared from rat spleen, peritoneal lavage cells, thymus, PBMCs, and human PBMCs, and transferred to PBS/0.5% BSA, where they were stained with multiple panels of fluorescent primary antibodies for 30 min. For the detection of surface markers, Hoechst 33258 fluorescent dye was added 5 min before measurement and used to exclude dead cells. To detect cytokines and transcription factors, fixable viability dyes were used to exclude dead cells. Subsequently, the cells were fixed and permeabilized using a Fixation and Permeabilization Kit (Thermo Fisher), following the manufacturer's instructions. They were then intracellularly stained with multiple panels of fluorescent primary antibodies. The complete list of antibodies used can be found in Supporting information Table S1. Rat flow cytometry data were collected using the LSR II cytometer (BD Bioscience) and analyzed using GateLogic 400.2A software (Invai). The gating strategy is shown in Supporting information Fig. S6. Human data were collected using the LSRFortessa Analyzer (BD Biosciences) and analyzed with FlowJo v10.0 (Tree Star). The gating strategy is shown in Supporting information Fig. S7.

Serum analysis

The rat blood was collected in tubes and left for 2 h at room temperature. Following this, it was centrifuged for 15 min at 1000 × *g*. The resulting serum was utilized for analysis. The concentration of norepinephrine was determined using an ELISA kit in accordance with the manufacturer's instructions (LS-F5550, LSBio). The concentration of cytokines in serum was measured by magnetic bead-based assays Bio-Plex Pro Rat Cytokine Th1/Th2 Assay (Bio-Rad) according to the manufacturer's instructions on the MAGPIX system (Luminex Corporation).

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Staining of reactive oxygen species, intracellular nitric oxide, and membrane potential of mitochondria

Splenocytes were stained for the detection of ROS production using 2′,7′-Dichlorofluorescein diacetate (DCFDA,15 μM; Sigma-Aldrich), for intracellular nitric oxide (NO) using diaminofluorescein-2 diacetate (DAF-2 DA; 2,5 μM; Abcam) or for mitochondrial membrane potential using MitoTracker Red CMXRos (100 nM; Thermo Fisher) according to the manufacturer's instructions for 30 min at 37°C. Poststaining, cells were washed in PBS/0.5% BSA, and surface markers were subsequently stained on ice.

Five minutes prior to measurement, Hoechst 33258 fluorescent dye was added to exclude dead cells. Data acquisition was performed using the LSR II cytometer and analyzed using GateLogic 400.2A software. The gating strategy is shown in Supporting information Fig. S8.

Bulk RNA-sequencing

γδ T cells from the spleen were FACS sorted directly into the lysis buffer of a RNeasy Micro Kit (QIAGEN). RNA was isolated immediately according to the manufacturer's instructions. The isolated RNA was then used for library preparation using the DNBSEQ Low Input Single Cell Smart-Seq Eukaryotic mRNA library preparation kit. Libraries were then sequenced on the BGI DNBseq platform in the PE100 regime. The reference mRatBN7.2 rat genome and annotation were downloaded from RefSeq (2023-05-29, https://www.ncbi.nlm.nih.gov/refseq/). Read quality was checked using FastQC version 0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adapters and low-quality sequences were removed using Trimmomatic 0.39 [65]. Clean reads were aligned to the rat genome using HISAT2 2.2.1 [66] and SAMtools 1.13 [67, 68]. Read coverage tracks were then computed and normalized to the respective mapped library sizes using deepTools 3.5.1 [69]. Mapped reads and coverage data were inspected visually in the IGV 2.9 browser [70]. Differentially expressed genes were detected using the DESeq2 packages [71] in R/Bioconductor [72, 73]. Gene-set enrichment analysis was performed using the WEB-based GEne SeT AnaLysis Toolkit (WebGestalt) [74]. Heatmaps were prepared using Phantasus application [75].

Processing of samples from human volunteers

The study involved 12 healthy individuals who regularly engage in cold water swimming, alongside a control group of 12 healthy volunteers. All procedures were performed following the acquisition of informed consent from the participants and in compliance with local ethical guidelines and the Helsinki Declaration. The demographic data of donors are listed in Supporting information Table S2. Peripheral blood samples were collected in 9 mL EDTA-coated tubes. PBMCs were isolated by Ficoll-Paque PLUS gradient centrifugation and used for immediate downstream cell analyses by flow cytometry. To determine cytokine production, 1 million PMBCs were cultured in 0.5 mL RPMI 1640 (supplemented with 10% fetal bovine serum) stimulated by LPS (1 μg/mL, Sigma-Aldrich) for 24 h. Subsequently, the supernatant was harvested and stored at −80°C, and the concentrations of TNF-α, IL-1β, IL-6, and IL-10 were determined via ELISA, following the manufacturer's instructions (R&D Systems).

Statistical analysis

Aside from RNA sequencing all plots and tests were generated using GraphPad Prism (GraphPad Software). Data are shown as mean with SD and all individual values or as boxes and whiskers (lines inside the boxes represent the median, and whiskers represent the minimum and maximum values). Human samples with donor gender displayed are shown as individual points with mean and SD. The distributions of data sets were tested by the Shapiro–Wilk test, determining the use of the parametric or nonparametric tests for subsequent analyses. Unpaired *t*-tests, as well as Mann–Whitney tests, were used to assess differences between the two groups. Differences among three or more groups were calculated using a one-way ANOVA followed by Holm-Sidak's test for multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

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Author contributions: Magdalena Krulova, Daniel Vasek, and Jitka M Zurmanova designed the study; Daniel Vasek, Peter Holicek, Frantisek Galatik, Anna Kratochvilova, Bianka Porubska, Veronika Somova, Natalie Fikarova, and Michaela Hajkova performed experiments; Martin Prevorovsky performed RNA-seq analysis; Daniel Vasek, Martin Prevorovsky, and Magdalena Krulova analyzed data; Magdalena Krulova and Daniel Vasek prepared the manuscript; Martin Prevorovsky and Jitka M Zur-

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manova critically revised the article. All authors read and approved the final manuscript.

Ethics statement and consent to participate: The experimental procedures involving live animals were performed in strict accordance with the Act No. 246/1992 coll., on the protection of animals against cruelty, the basic law related to animal protection governing the activities of all the state authorities of animal protection in the Czech Republic. This study protocol was reviewed and approved by the expert commission for ensuring the welfare of experimental animals, Faculty of Science, Charles University approval number MSMT-31167/2021-4. Additionally, the approval was granted by the Ethics Committee of the Faculty of Science, Charles University, Prague, Czech Republic (Date: 28. 2. 2022, Approval number: 2022).

Written informed consent was obtained from all study participants according to the ethical guidelines of the Declaration of Helsinki.

Data availability statement: All scripts used for RNA-seq data analysis are available at https://github.com/mprevorovsky/krulova-rats-cold. The raw and processed RNA-seq data are available from the ArrayExpress database (https://www.ebi.ac.uk/biostudies/arrayexpress) under the accession number E-MTAB-13420.

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Abbreviations: **AR**: adrenergic receptor · **BAT**: brown adipose tissue · **DAF-2 DA**: diaminofluorescein-2 diacetate · **DCFDA**: 2′,7′-Dichlorofluorescein diacetate · **ILC2**: innate lymphoid cell type 2 · **NE**: norepinephrine · **NO**: nitric oxide · **UCP1**: uncoupling protein 1 · **WAT**: white adipose tissue

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Supplementary material

Tab. S1 List of antibodies and reagents used for flow cytometry

Parameter	Specificity	Producer	Clone	Fluorochrome	Dilution
CD45	anti-rat	BioLegend	OX-1	AF700	1:200
CD45	anti-rat	SONY	OX-1	FITC	1:200
CD3	anti-rat	SONY	1F4	APC	1:100
CD45RA	anti-rat	BioLegend	OX-33	APC	1:80
CD45RA	anti-rat	SONY	OX-33	FITC	1:80
CD11b/c	anti-rat	BioLegend	OX-42	APC	1:80
CD11b/c	anti-rat	SONY	OX-42	PE	1:80
CD80	anti-rat	BD Biosciences	3H5	PE	1:100
γδTCR	anti-rat	SONY	V65	PE	1:100
TNFα	anti-rat	Invitrogen	TN3-19.12	FITC	1:100
IL-17A	anti-rat	Invitrogen	eBio17B7	APC	1:80
CD68	anti-rat	BioLegend	QA20A71	PE	1:100
CD163	anti-rat	Bio-Rad	ED2	FITC	1:80
Ki67	anti-rat	Invitrogen	SolA15	FITC	1:100
CD45	anti-human	BioLegend	HI30	PB	1:100
CD45	anti-human	BD Biosciences	HI30	HV500	1:100
CD3	anti-human	BioLegend	OKT3	AF700	1:100
CD20	anti-human	EXBIO	LT20	FITC	1:100
CD56	anti-human	BioLegend	MEM-188	PerCP-Cy5.5	1:100
CD33	anti-human	BioLegend	WM53	PE	1:100
CD14	anti-human	BioLegend	LM2	Texas Red	1:100
CD11b	anti-human	Beckman Coulter	RM052	PB	1:100
CD11c	anti-human	EXBIO	BU-15	APC	1:100
CD4	anti-human	Beckman Coulter	SFCI12T4D11	ECD	1:100
CD8	anti-human	BD Biosciences	RPA-T8	HV500	1:100
γδTCR	anti-human	SONY	B1	PE-Cy7	1:100
RORγt	anti-human	BD Biosciences	AFKJS-9	PE	1:100
T-bet	anti-human	BD Biosciences	eBio4B10	e660	1:100
Reagents used to differentiate live/dead cells					
Reagent			Producer		
Hoechst 33258 fluorescent dye			Sigma-Alrich		
Propidium iodide			EXBIO		
LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit			Thermo Fisher		
Fixable Viability Dye eFluor™ 780			BD Biosciences		

Tab. S2 The main characteristics of all human volunteers

Volunteers	Regular cold swimmers group	Control group
Number (sex m / f)	n = 12 (9 / 3)	n = 12 (4 / 8)
Age [year]	median 38 (27-57)	median 30 (21-58)
BMI [kg/m^2]	median 24,27 (20,37-28,96)	median 24,8 (17,7-31,31)
Duration of cold swimming [year]	mean 5,7 / median 3,5	-
Frequency of cold swimming [days per week]	mean 4,7 / median 5	-

Fig. S1

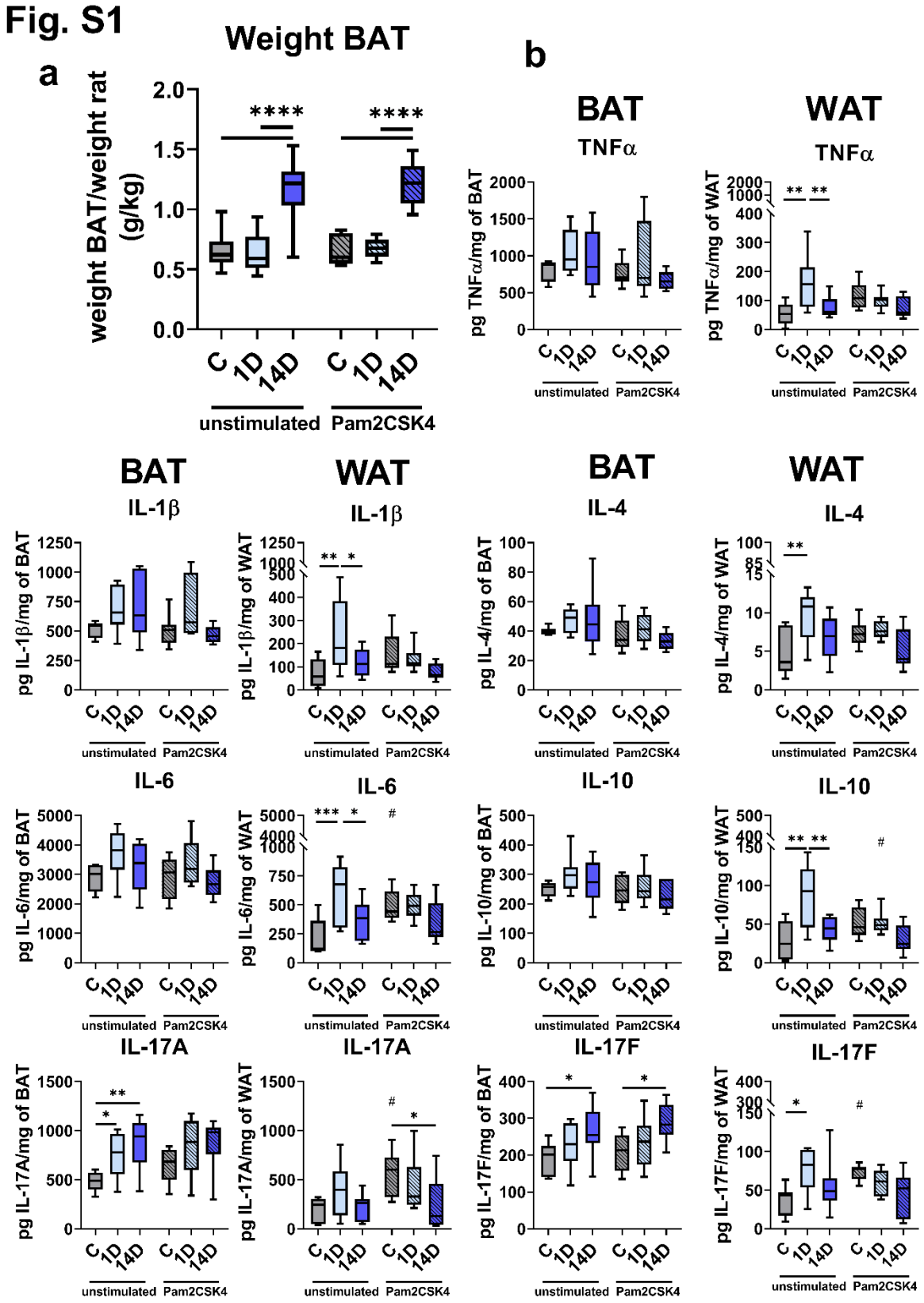

a
Weight BAT
weight BAT/weight rat (g/kg)
****
****
C 1D 14D C 1D 14D
unstimulated Pam2CSK4
b
BAT
TNFα
WAT
TNFα
pg TNFα/mg of BAT
pg TNFα/mg of WAT
BAT
IL-1β
WAT
IL-1β
pg IL-1β/mg of BAT
pg IL-1β/mg of WAT
BAT
IL-4
WAT
IL-4
pg IL-4/mg of BAT
pg IL-4/mg of WAT
IL-6
IL-6
pg IL-6/mg of BAT
pg IL-6/mg of WAT
IL-10
IL-10
pg IL-10/mg of BAT
pg IL-10/mg of WAT
IL-17A
IL-17A
pg IL-17A/mg of BAT
pg IL-17A/mg of WAT
IL-17F
IL-17F
pg IL-17F/mg of BAT
pg IL-17F/mg of WAT
unstimulated
Pam2CSK4

Fig. S1 Changes in adipose tissue after TRL2 stimulation. **a-b** Rats exposed to 1 day (1D) or 14 days (14D) of cold or the controls (C) were challenged with Pam2CSK4. **a** Brown adipose tissue (BAT) was isolated from the interscapular area and weighted. *n* = 9-16. **b** The homogenate was prepared from BAT and white adipose tissue (WAT) and concentration of cytokines TNFα, IL-1β, IL-4, IL-6, IL-10, IL-17A and IL-17F were determined by ELISA. Data are shown as boxes and whiskers. Lines inside the boxes represent the median, and whiskers represent the minimum and maximum values. The level of statistical significance was determined using ANOVA followed by Holm-Sidak´s test for multiple comparisons *n* = 6-12 ($^{x}p < 0.05$, $^{xx}p < 0.01$, $^{xxx}p < 0.001$) * indicates significance within the same group. # indicates significance between groups

Fig. S2

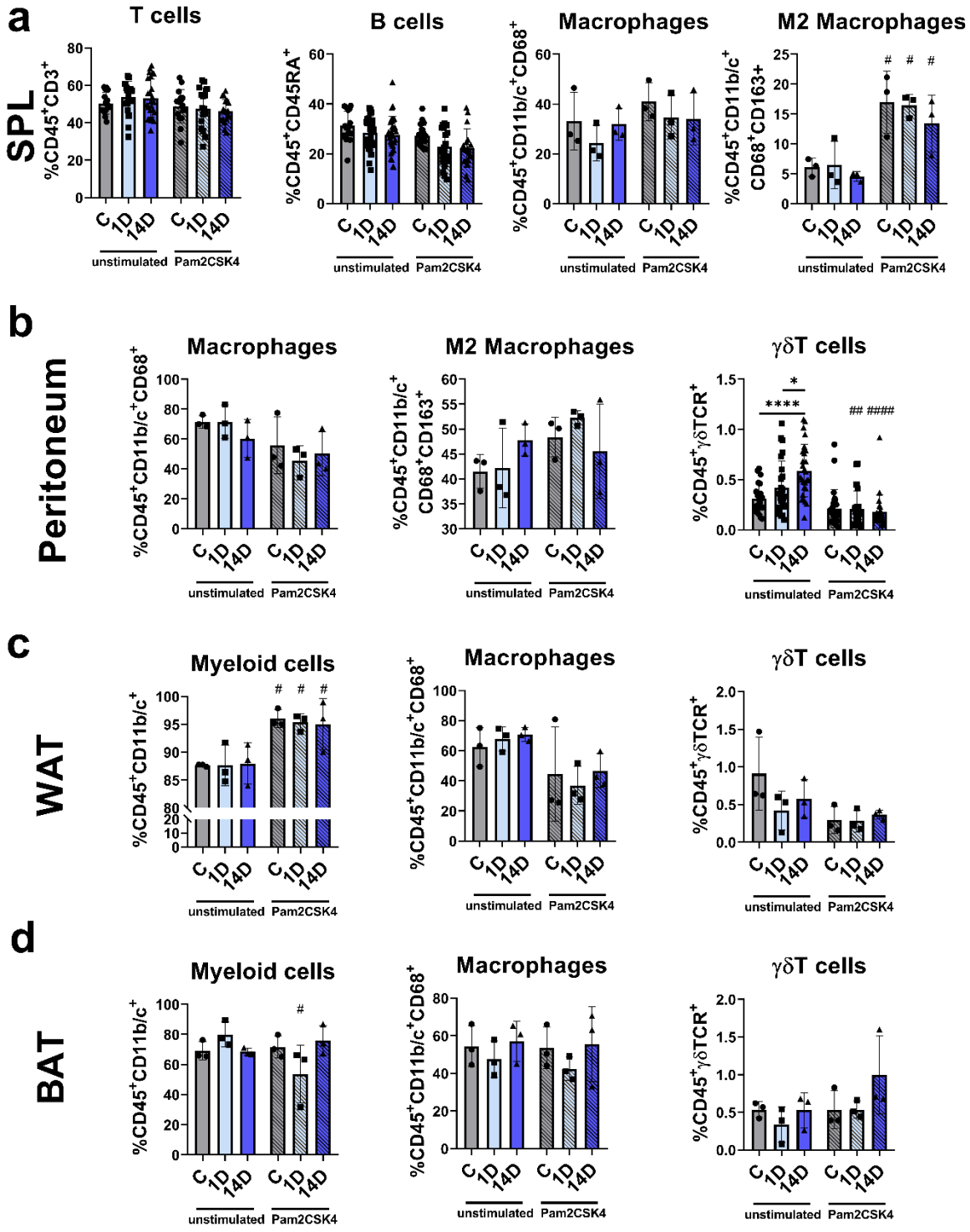


Fig. S2 Representation of splenic immune populations after TLR2 stimulation. Rats exposed to 1 day (1D) or 14 days (14D) of cold or the controls (C) were challenged with Pam2CSK4. **a** The representation of T cells ($CD45^+CD3^+$), B cells ($CD45^+CD45RA^+$), macrophages ($CD45^+CD11b/c^+CD68^+$), and M2 macrophages ($CD45^+CD11b/c^+CD68^+CD163^+$) in spleen were determined by flow cytometry. **b-d** The representation of myeloid cells ($CD45^+CD11b/c^+$),

macrophages (CD45$^+$CD11b/c$^+$CD68$^+$), and γδ T cells (CD45$^+$γδTCR$^+$) in peritoneum (**b**), white adipose tissue (**c**), and brown adipose tissue (**d**) were determined by flow cytometry. Data are shown as means with individual values ± SD. The level of statistical significance was determined using ANOVA followed by Holm-Sidak´s test for multiple comparisons *n* = 14 (^{x}p < 0.05, ^{xxxx}p < 0.0001) * indicates significance within the same group. # indicates significance between groups

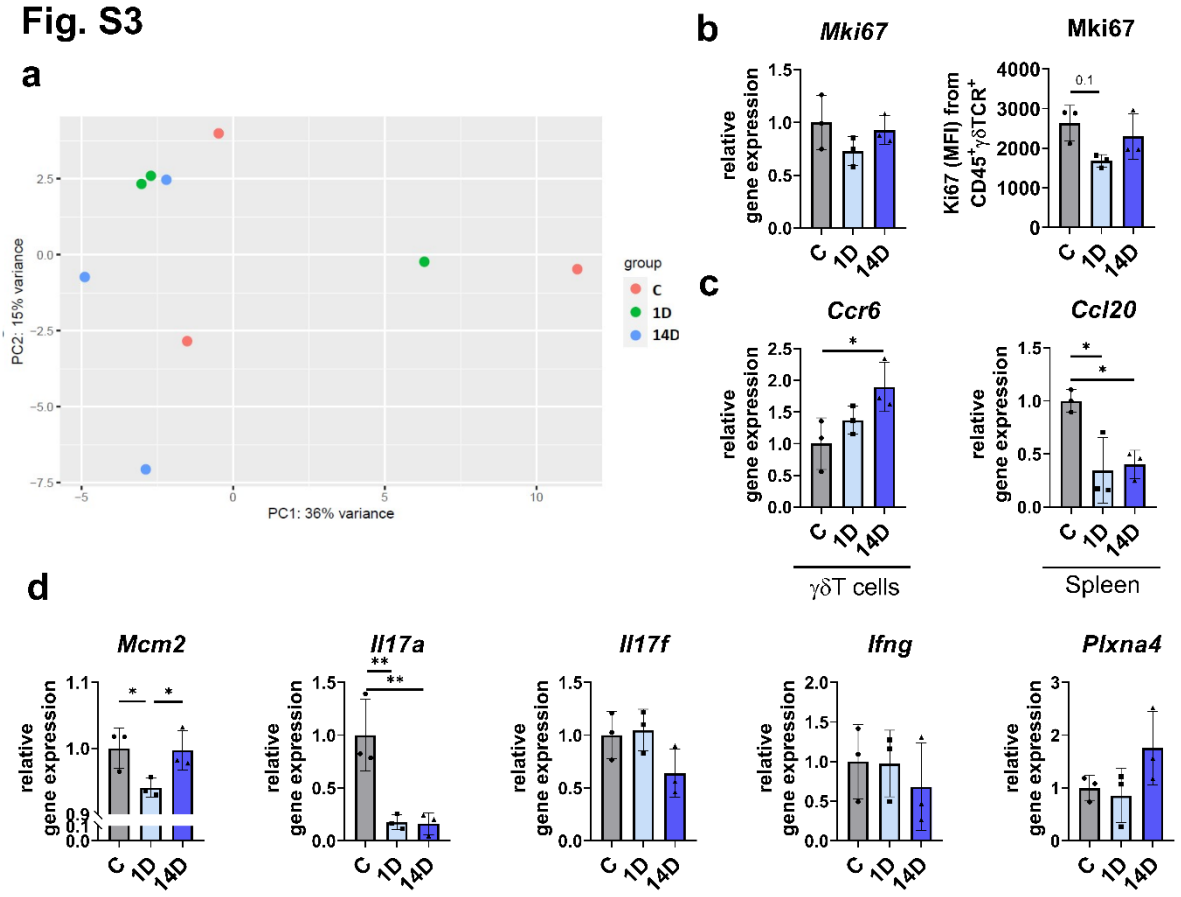


Fig. S3 Validation of RNA-seq. **a-d** FACS-sorted splenic γδ T cells isolated from control (C), 1 day (1D) or 14 days (14D) cold-exposed rats were analyzed. **a** PCA dataset obtained from bulk RNA-sequencing of isolated γδ T cells. **b** Relative gene expression of *Mki67* from FACS-sorted splenic γδ T cells was determined by qPCR (left) and mean fluorescence intensity (MFI) of protein Ki67 gated from CD45$^+$γδTCR$^+$ in spleen (right) was determined by FACS. **c** Relative gene expression of *Ccr6* from FACS-sorted splenic γδ T cells and *Ccl20* from whole spleen were determined by qPCR. **d** Relative gene expression of *Mcm2*, *Il17a*, *Il17f*, *Ifng,* and *Plxna4* from FACS-sorted splenic γδ T cells were determined by qPCR. **b-d** γδ T cells from the spleen were FACS sorted directly into the lysis buffer of a RNeasy® Micro Kit (QIAGEN). RNA was isolated immediately according to the manufacturer's instructions. Reverse transcription was performed with SuperScript™ IV Reverse Transcriptase (Thermo Fisher) according to the manufacturer's instructions, including RNaseOUT™ Recombinant Ribonuclease Inhibitor (Thermo Fisher) and

Random Hexamer Primer (Thermo Fisher). qPCR was performed by using HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne) according to the manufacturer's instructions and measured by LightCyler480 (Roche). *B2m* and *Rps18* were used as housekeeping genes. The primers are detailed in Supplementary Table S3. Data are shown as means with individual values ± SD. The level of statistical significance was determined using ANOVA followed by Holm-Sidak´s test for multiple comparisons *n=3* (*p<0.05, **p<0.01)

Tab. S3 of used primers for qPCR.

***Rattus norvegicus* gene symbol**	**Official Full Name**	***Rattus norvegicus* protein symbol**	**RGD ID**	**Primer sequence**
Mki67	marker of proliferation Ki-67	Ki67	1305476	ACCGCCTCAGAGATTTTGGG
				TGTTTGATGCTCTGCCGTCT
Ccr6	C-C motif chemokine receptor 6	CCR6	1308562	AACCCCGTGTTGTATGCCTT
				TTCTGAGTAAACCCGGGCAC
Ccl20	C-C motif chemokine ligand 20	CCL20	3646	TGCTGCCTCACGTACACAAA
				CAGCGCACACGGATCTTTTC
Mcm2	minichromosome maintenance complex component 2	Mcm2	1305577	AACCCAGGTGGAAAGCACAA
				TGGTGAAAATGGCACGGCTA
Il17a	interleukin 17A	IL-17A	2888	CCGAGATAACTTTGAGGCATA
				AACGAGGTTTGACTTTCACA
Il17f	interleukin 17F	IL-17F	1311398	GGAAAAGCCTCCTTTGATCC
				ACGGAGCTTCAAGGATGTTG
Ifng	interferon gamma	IFN	2866	TGAAAGCCTAGAAAGTCTGAAGAACTA
				CGTGTTACCGTCCTTTTGC
Plxna4	plexin A4	Plxna4	1308074	GGGATGGGTTCCTCTACCCT
				AAGGTGATTGAAGCCCTCGG
B2m	beta-2 microglobulin	B2m	2189	AGCAGGTTCCTCAAACAAGG
				TTCTGCCTTGGAGTCCTTTC
Rps18	ribosomal protein S18	Rps18	3595	TTCAGCACATCCTGCGAGTA
				TTGGTGAGGTCAATGTCTG

Tab. S4

a

1D vs C

Group of genes	Gene Symbol	Score	Gene Name	Gene ID
Inorganic cation transport	Cacna6	4.7063	calcium voltage-gated channel auxiliary subunit gamma 6	140727
	Kcnk4	4.3294	potassium two pore domain channel subfamily K member 4	116489
	Nalcn	3.8631	sodium leak channel, non-selective	266760
	Slc24a4	3.7838	solute carrier family 24 member 4	314396
	Slc41a2	3.5787	solute carrier family 41 member 2	362861
	Trpm8	3.4361	transient receptor potential cation channel, subfamily M, member 8	171384
	Cacna1s	3.2455	calcium voltage-gated channel subunit alpha1 S	682930
	Slc11a1	3.1173	solute carrier family 11 member 1	316519
	Atp2b2	2.987	ATPase plasma membrane Ca2+ transporting 2	24215
	Atp2b2	2.987	ATPase plasma membrane Ca2+ transporting 2	24215
	Ednra	2.8629	endothelin receptor type A	24326
	Gramd2a	2.8389	GRAM domain containing 2A	300761
	Iph2	2.7489	junctophilin 2	296345
	Adora1	2.5074	adenosine A1 receptor	29290
	Ptgs2	2.4655	prostaglandin-endoperoxide synthase 2	29527
	Scn1b	2.436	sodium voltage-gated channel beta subunit 1	29686
	Stac3	2.3749	SH3 and cysteine rich domain 3	362895
	Cacnb4	2.3704	calcium voltage-gated channel auxiliary subunit beta 4	58942
	Kcnj2	2.0114	potassium voltage-gated channel subfamily J member 2	29712
	Ccl24	1.9978	C-C motif chemokine ligand 24	288593
	Grin3a	1.8803	glutamate ionotropic receptor NMDA type subunit 3A	191573
	P2rx1	1.6477	purinergic receptor P2X 1	25505
	Scn3b	1.601	sodium voltage-gated channel beta subunit 3	245956
	Scn3a	1.4099	sodium voltage-gated channel alpha subunit 3	497770
	P2ry12	0.9827	purinergic receptor P2Y12	64803
Response to chemokine	Cxcr2	4.6419	C-X-C motif chemokine receptor 2	29385
	Tlr4	4.2300	toll-like receptor 4	29260
	Cxcl11	3.7053	C-X-C motif chemokine ligand 11	305236
	Il1a	2.7571	interleukin 1 alpha	24493
	Ccl9	1.935	chemokine (C-C motif) ligand 9	360579
	Ccl22	1.9341	C-C motif chemokine ligand 22	117551
	Cxcl2	1.8499	C-X-C motif chemokine ligand 2	114105
	Cxcl13	1.6564	C-X-C motif chemokine ligand 13	498335
Lipid catabolic process	Cyp39a1	4.5999	cytochrome P450, family 39, subfamily a, polypeptide 1	301264
	Lipg	3.0488	lipase G, endothelial type	291437
	Apoa5	2.4803	apolipoprotein A5	140638
	Pla2g2a	2.3393	phospholipase A2 group IIA	29692
	Pla1a	2.301	phospholipase A1 member A	85311
	Pla2g5	2.1119	phospholipase A2, group V	29354
	Pla2g7	1.6994	phospholipase A2 group VII	301265
	Pla2g10	1.5666	phospholipase A2, group X	29359
	Plcb4	1.2034	phospholipase C, beta 4	25031
Oxidative phosphorylation	Cox4i2	4.2937	cytochrome c oxidase subunit 4i2	84683
	Cox8b	2.8548	cytochrome c oxidase, subunit VIIIb	25250
	Atp6v1b1	2.4268	ATPase H+ transporting V1 subunit B1	312488
	Atp6v1g3	1.5988	ATPase H+ transporting V1 subunit G3	289407
	ATP8	1.2841	ATPase subunit 8	26196
	ND5	1.1726	NADH dehydrogenase subunit 5	26202
	Atp4a	1.142	ATPase H+/K+ transporting subunit alpha	24216
	ND6	1.1391	NADH dehydrogenase subunit 6	26203
	COX3	1.1251	cytochrome c oxidase subunit 3	26204
	COX2	1.0089	COXII	26198
	COX1	0.9235	cytochrome c oxidase subunit 1	26195
DNA replication & Cell cycle	Tgfb3	-2.885	transforming growth factor, beta 3	25717
	Cdkn1a	-1.9231	cyclin-dependent kinase inhibitor 1A	114851
	Cdc6	-1.235	cell division cycle 6	360621
	Ccnd1	-0.9231	cyclin D1	58919
	Exo1	-0.8797	exonuclease 1	305000
	Ccna1	-0.7759	cyclin A1	295052
	Gadd45g	-0.7344	growth arrest and DNA-damage-inducible, gamma	291005
	Orc1	-0.678	origin recognition complex, subunit 1	313479
	LOC100364016	-0.6323	100364016	
	Pole2	-0.6262	DNA polymerase epsilon 2, accessory subunit	299112
	E2f1	-0.6023	E2F transcription factor 1	399489
	Ccnd2	-0.5317	cyclin D2	64033
	Ywhag	-0.5084	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma	56010
	Ccne1	-0.4665	cyclin E1	25729
	Tgfb1	-0.4332	transforming growth factor, beta 1	59086
	Mcm4	-0.3947	minichromosome maintenance complex component 4	29728
	Mcm2	-0.3878	minichromosome maintenance complex component 2	312538
	Cdc20	-0.3179	cell division cycle 20	64515
	Rnaseh1	-0.3176	ribonuclease H1	298933
	Tfdp1	-0.3157	transcription factor Dp-1	361178
	Gadd45b	-0.2796	growth arrest and DNA-damage-inducible, beta	299626
	Pold1	-0.2783	DNA polymerase delta 1, catalytic subunit	59294
	Rfc2	-0.2774	replication factor C subunit 2	116468
	Rpa1	-0.2454	replication protein A1	287524
	Mcm7	-0.2332	minichromosome maintenance complex component 7	288532

b

14D vs C

Group of genes	Gene Symbol	Score	Gene Name	Gene ID
Second-messenger-mediated signaling, Adrenergic receptors	Ghrh	5.263	growth hormone releasing hormone	29446
	Ptgir	5.0027	prostaglandin I2 receptor	292661
	Adgrg7	4.6405	adhesion G protein-coupled receptor G7	304014
	Nppa	3.1649	natriuretic peptide A	24602
	Hpca	2.845	hippocalcin	29177
	Adra2a	2.7587	adrenoceptor alpha 2A	25083
	Adm2	0.9169	adrenomedullin 2	399475
	Adrb3	0.9169	adrenoceptor beta 3	25645
Antigen processing and presention	RT1-M5	4.8868	RT1 class Ib, locus M5	499400
	Hspa1l	1.7623	heat shock protein family A (Hsp70) member 1 like	24963
	Klrc1	1.4968	killer cell lectin like receptor C1	29683
	RT1-A1	1.4364	RT1 class Ia, locus A1	24973
	Klrc3	1.1242	killer cell lectin like receptor C3	500338
	Psme3	1.085	proteasome activator subunit 3	287716
	Klrc2	1.0001	killer cell lectin like receptor C2	29684
	RT1-Bb	0.9449	RT1 class II, locus Bb	309622
	RT1-CE16	0.8943	RT1 class I, locus CE16	414819
	RT1-A3	0.8747	RT1 class I, locus A3	309627
Semaphorins & Sphingosine-1-phosphate receptor 3	S1pr3	2.9256	sphingosine-1-phosphate receptor 3	306792
	Sema3e	2.4012	semaphorin 3E	296789
	Trem2	1.723	triggering receptor expressed on myeloid cells 2	301227
	Nrp1	1.4124	neuropilin 1	246331
	Pak1	1.1565	p21 (RAC1) activated kinase 1	29431
	Sema6d	1.0664	semaphorin 6D	311384
	Plxna4	1.008	plexin A4	312213
	Sema3a	1.0044	semaphorin 3A	29751
Glutamate receptors	Grm2	3.1674	glutamate metabotropic receptor 2	24415
	Grm3	1.8608	glutamate metabotropic receptor 3	24416
	Grin2a	1.2429	glutamate ionotropic receptor NMDA type subunit 2A	24409
	Grm1	1.1509	glutamate receptor, metabotropic 1	24414
	Grin2d	1.1397	glutamate ionotropic receptor NMDA type subunit 2D	24412
	Grik3	0.9169	glutamate ionotropic receptor kainate type subunit 3	298521
Acetylcholine receptors	Chrna9	3.1571	cholinergic receptor nicotinic alpha 9 subunit	65024
	Chrna4	2.8514	cholinergic receptor nicotinic alpha 4 subunit	25590
	Chrm1	1.1251	cholinergic receptor, muscarinic 1	25229
IL-17 signaling pathway	Il17f	-3.7373	interleukin 17F	301291
	S100a9	-3.0863	S100 calcium binding protein A9	94195
	Ccl12	-1.9042	chemokine (C-C motif) ligand 12	287562
	Cxcl3	-1.9042	chemokine (C-X-C motif) ligand 3	171551
	Il6	-1.9042	interleukin 6	24498
	Il17a	-1.782	interleukin 17A	301289
	Fosb	-1.2391	FosB proto-oncogene, AP-1 transcription factor subunit	100360880
	Mmp13	-1.0608	matrix metallopeptidase 13	171052
	Ccl17	-1.0067	C-C motif chemokine ligand 17	117518
	Cxcl1	-1.0067	C-X-C motif chemokine ligand 1	81503
	Mapk13	-1.0067	mitogen activated protein kinase 13	29513
	Mapk4	-1.0067	mitogen-activated protein kinase 4	54268
	Mmp1	-1.0067	matrix metallopeptidase 1	300339
	Mapk10	-0.9528	mitogen activated protein kinase 10	25272
	Tnfaip3	-0.8798	TNF alpha induced protein 3	683206
	Il17d	-0.7633	interleukin 17D	691799
	Jun	-0.7387	Jun proto-oncogene, AP-1 transcription factor subunit	24516
	Nfkbia	-0.7148	NFKB inhibitor alpha	25493
	Cxcl10	-0.7057	C-X-C motif chemokine ligand 10	245920
	Mapk11	-0.5725	mitogen-activated protein kinase 11	689314
	Il1b	-0.5148	interleukin 1 beta	24494
	Ifng	-0.514	interferon gamma	25712
	Fos	-0.4998	Fos proto-oncogene, AP-1 transcription factor subunit	314322
cytochrome P450	Cyp26c1	-3.5403	cytochrome P450, family 26, subfamily C, polypeptide 1	308190
	Aox2	-3.2589	aldehyde oxidase 2	316421
	Cyp2a2	-2.837	cytochrome P450, family 2, subfamily a, polypeptide 2	24895
	Cyp2c6v1	-2.4613	cytochrome P450, family 2, subfamily C, polypeptide 6, variant 1	293989
	Ugt2b17	-2.4432	UDP glucuronosyltransferase 2 family, polypeptide B15	266685
	Cyp3a18	-2.2477	cytochrome P450, family 3, subfamily a, polypeptide 18	252931
	Bco1	-2.2304	beta-carotene oxygenase 1	114106
	Hsd17b6	-1.9042	hydroxysteroid (17-beta) dehydrogenase 6	286964
	Aldh1a2	-1.4689	aldehyde dehydrogenase 1 family, member A2	116676
	Cyp2c22	-1.4391	cytochrome P450, family 2, subfamily c, polypeptide 22	171518
	P2rx1	-1.0898	purinergic receptor P2X 1	25505
	Cyp3a62	-1.0729	cytochrome P450, family 3, subfamily a, polypeptide 62	170509

c

14D vs 1D

Group of genes	Gene Symbol	Score	Gene Name	Gene ID
Cell cycle	Cdkn2b	3.1801	cyclin-dependent kinase inhibitor 2B	25164
	Cdkn2a	2.868	cyclin-dependent kinase inhibitor 2A	25163
	Cdkn1a	1.7853	cyclin-dependent kinase inhibitor 1A	114851
	Ccnd1	1.6502	cyclin D1	58919
	Cdc6	1.4386	cell division cycle 6	360621
	Tgfb3	1.4151	transforming growth factor, beta 3	25717
	Orc1	0.7473	origin recognition complex, subunit 1	313479
	Cdkn1c	0.7226	cyclin-dependent kinase inhibitor 1C	246060
	LOC100364016	0.7176	100364016	
	Gadd45e	0.6888	growth arrest and DNA-damage-inducible, gamma	291005
	Fzr1	0.6289	fizzy and cell division cycle 20 related 1	314642
	Ccna2	0.5372	cyclin A2	114494
	Ccnd2	0.5253	cyclin D2	64033
	Ska1	1.2121	spindle and kinetochore associated complex subunit 1	291441
	Kntc1	1.2091	kinetochore associated 1	304477
	Spc2	1.1287	shugoshin 2	316425
	Kif2c	1.0935	kinesin family member 2C	171529
	Kif18a	1.0156	kinesin family member 18A	362186
	Cenph	0.875	centromere protein H	681185
	Cenpu	0.8228	centromere protein U	304464
	Psme3	0.782	proteasome activator subunit 3	287716
	Cdt1	0.7275	chromatin licensing and DNA replication factor 1	292071
Semaphorins & Sphingosine-1-phosphate receptor 3	S1pr3	3.6958	sphingosine-1-phosphate receptor 3	306792
	Sema3a	2.8041	semaphorin 3A	29751
	Cdk5r1	1.356	cyclin-dependent kinase 5 regulatory subunit 1	116671
	Plxna4	1.056	plexin A4	312213
	Plxna3	1.0426	plexin A3	309280
	Plxna2	0.9399	plexin A2	289392
Cell adhesion mediated by integrin	Itga5	4.6398	integrin subunit alpha 5	315346
	Itgb1	2.6747	integrin subunit beta like 1	498564
	Nexmif	1.826	neurite extension and migration factor	302396
	Hrg	1.8212	histidine-rich glycoprotein	171016
	Itgb6	0.9399	integrin subunit beta 6	311061
Butyrophilin (BTN) family interaction	Cd209	2.868	CD209 molecule	363856
	Btnl2	2.8468	butyrophilin-like 2	309620
	Cd209e	2.4522	CD209e molecule	501797

Tab. S4 Table of gene-set enrichment analysis. **a-c** The table shows the enrichment scores of the important enriched and suppressed genes in the individual comparisons. The bulk RNA-sequencing analysis of FACS-sorted splenic γδ T cells isolated from control (C), 1 day (1D) or 14 days (14D) cold-exposed rats was performed. DESeq2 calculated $\log_2$ fold changes for 1D vs. C (**a**), 14D vs. C (**b**), and 14D vs. 1D (**c**) was used as input and analyzed for enrichment of pathways using GO and rno resource

Fig. S4

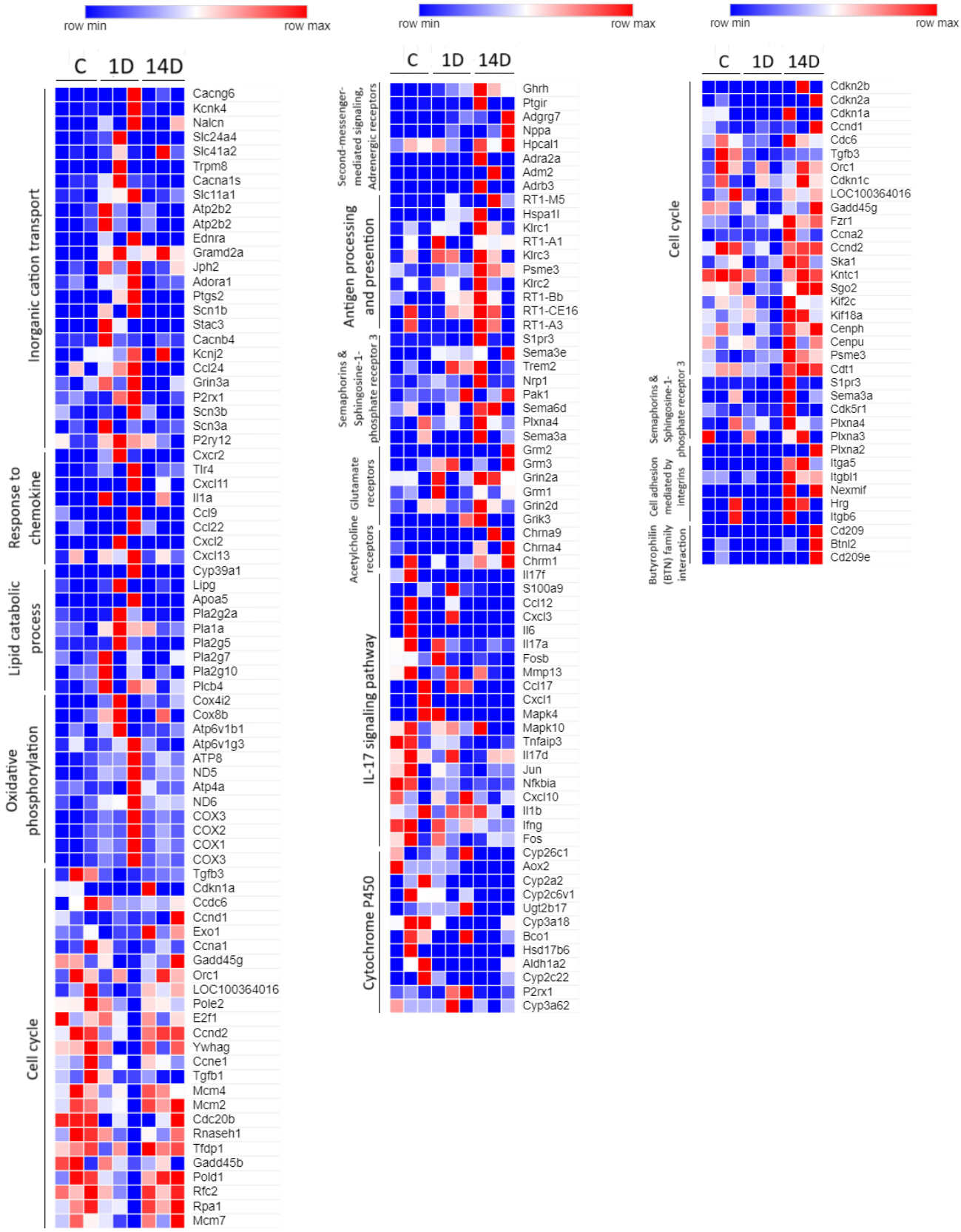

a
b
c
row min
row max
C
1D
14D
Inorganic cation transport
Response to chemokine
Lipid catabolic process
Oxidative phosphorylation
Cell cycle
Cacng6
Kcnk4
Nalcn
Slc24a4
Slc41a2
Trpm8
Cacna1s
Slc11a1
Atp2b2
Atp2b2
Ednra
Gramd2a
Jph2
Adora1
Ptgs2
Scn1b
Stac3
Cacnb4
Kcnj2
Ccl24
Grin3a
P2rx1
Scn3b
Scn3a
P2ry12
Cxcr2
Tlr4
Cxcl11
Il1a
Ccl9
Ccl22
Cxcl2
Cxcl13
Cyp39a1
Lipg
Apoa5
Pla2g2a
Pla1a
Pla2g5
Pla2g7
Pla2g10
Plcb4
Cox4i2
Cox8b
Atp6v1b1
Atp6v1g3
ATP8
ND5
Atp4a
ND6
COX3
COX2
COX1
COX3
Tgfb3
Cdkn1a
Ccdc6
Ccnd1
Exo1
Ccna1
Gadd45g
Orc1
LOC100364016
Pole2
E2f1
Ccnd2
Ywhag
Ccne1
Tgfb1
Mcm4
Mcm2
Cdc20b
Rnaseh1
Tfdp1
Gadd45b
Pold1
Rfc2
Rpa1
Mcm7
Second-messenger-mediated signaling, Adrenergic receptors
Antigen processing and presention
Semaphorins & Sphingosine-1-phosphate receptor 3
Glutamate receptors
Acetylcholine receptors
IL-17 signaling pathway
Cytochrome P450
Ghrh
Ptgir
Adgrg7
Nppa
Hpcal1
Adra2a
Adm2
Adrb3
RT1-M5
Hspa1l
Klrc1
RT1-A1
Klrc3
Psme3
Klrc2
RT1-Bb
RT1-CE16
RT1-A3
S1pr3
Sema3e
Trem2
Nrp1
Pak1
Sema6d
Plxna4
Sema3a
Grm2
Grm3
Grin2a
Grm1
Grin2d
Grik3
Chrna9
Chrna4
Chrm1
Il17f
S100a9
Ccl12
Cxcl3
Il6
Il17a
Fosb
Mmp13
Ccl17
Cxcl1
Mapk4
Mapk10
Tnfaip3
Il17d
Jun
Nfkbia
Cxcl10
Il1b
Ifng
Fos
Cyp26c1
Aox2
Cyp2a2
Cyp2c6v1
Ugt2b17
Cyp3a18
Bco1
Hsd17b6
Aldh1a2
Cyp2c22
P2rx1
Cyp3a62
Cell cycle
Semaphorins & Sphingosine-1-phosphate receptor 3
Cell adhesion mediated by integrins
Butyrophilin (BTN) family interaction
Cdkn2b
Cdkn2a
Cdkn1a
Ccnd1
Cdc6
Tgfb3
Orc1
Cdkn1c
LOC100364016
Gadd45g
Fzr1
Ccna2
Ccnd2
Ska1
Kntc1
Sgo2
Kif2c
Kif18a
Cenph
Cenpu
Psme3
Cdt1
S1pr3
Sema3a
Cdk5r1
Plxna4
Plxna3
Plxna2
Itga5
Itgbl1
Nexmif
Hrg
Itgb6
Cd209
Btnl2
Cd209e

Fig. S4 Heatmaps of important genes. **a-c** Heatmaps shows normalized counts reads of individual samples from table of gene-set enrichment analysis (**Tab S3**). The bulk RNA-sequencing analysis of FACS-sorted splenic γδT cells isolated from control (C), 1 day (1D) or 14 days (14D) cold-exposed rats was performed

Fig. S5

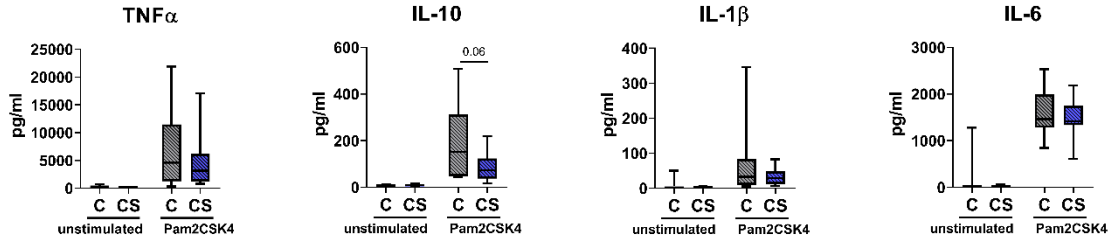


Fig. S5 Cytokine production by regular cold swimmers. PBMCs were isolated from human volunteers who regularly swim in cold water (cold swimmers - CS) and healthy controls (C). Cells were cultivated w/o Pam2CSK4 for 24 hours and concentration of cytokines TNFα, IL-10, IL-1β, and IL-6 was determined by ELISA. The data is depicted using box-and-whisker plots, where medians are indicated by lines inside the boxes, and whiskers represent the range of values. a-c The data was tested by the Mann-Whitney test or Unpaired t-test, since it passed the Shapiro-Wilk normality test. *n = 12*

Fig. S6

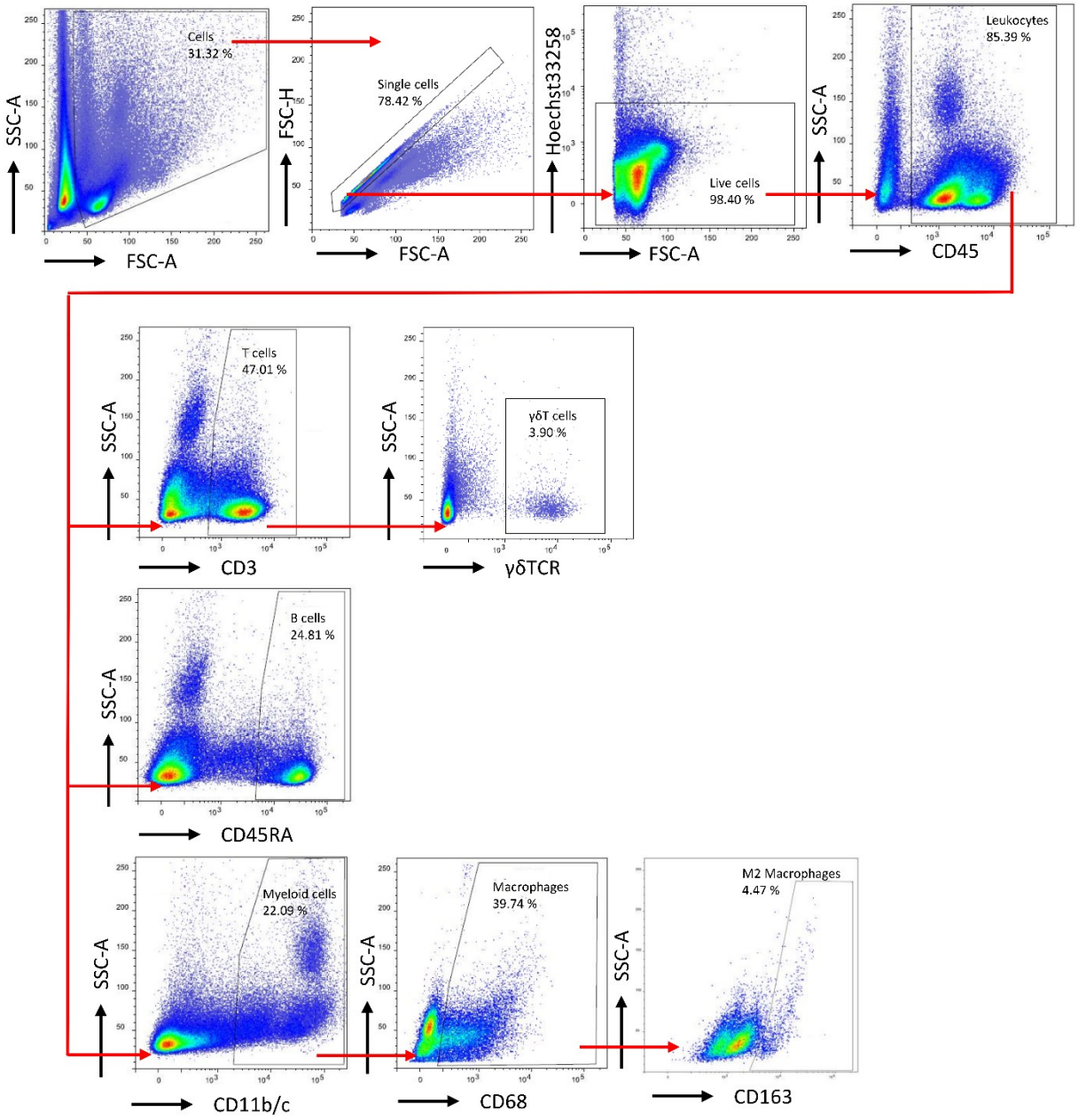


Fig. S6 Gating strategy for determining immune population. Single cell suspension was prepared from different organs. Afterwards, expression specific markers was determined by flow cytometry. Gating strategy: debris was excluded based on forward and side scatter (FSC, SSC), then doublets were excluded, and live cells were gated based on Hoechst 33258 negative population (Live cells). Leukocytes were selected based on CD45 positivity. From leukocytes were selected T cells based on CD3 positivity and γδ T cells were subsequently selected from them based on γδTCR positivity. B cells were selected from leukocytes based on CD45RA positivity and Myeloid cells were selected from leukocytes based on CD11b/c positivity. From the myeloid cells, macrophages were determined based on the expression of CD68 and from them the M2 subtype based on the expression of CD163

Fig. S7

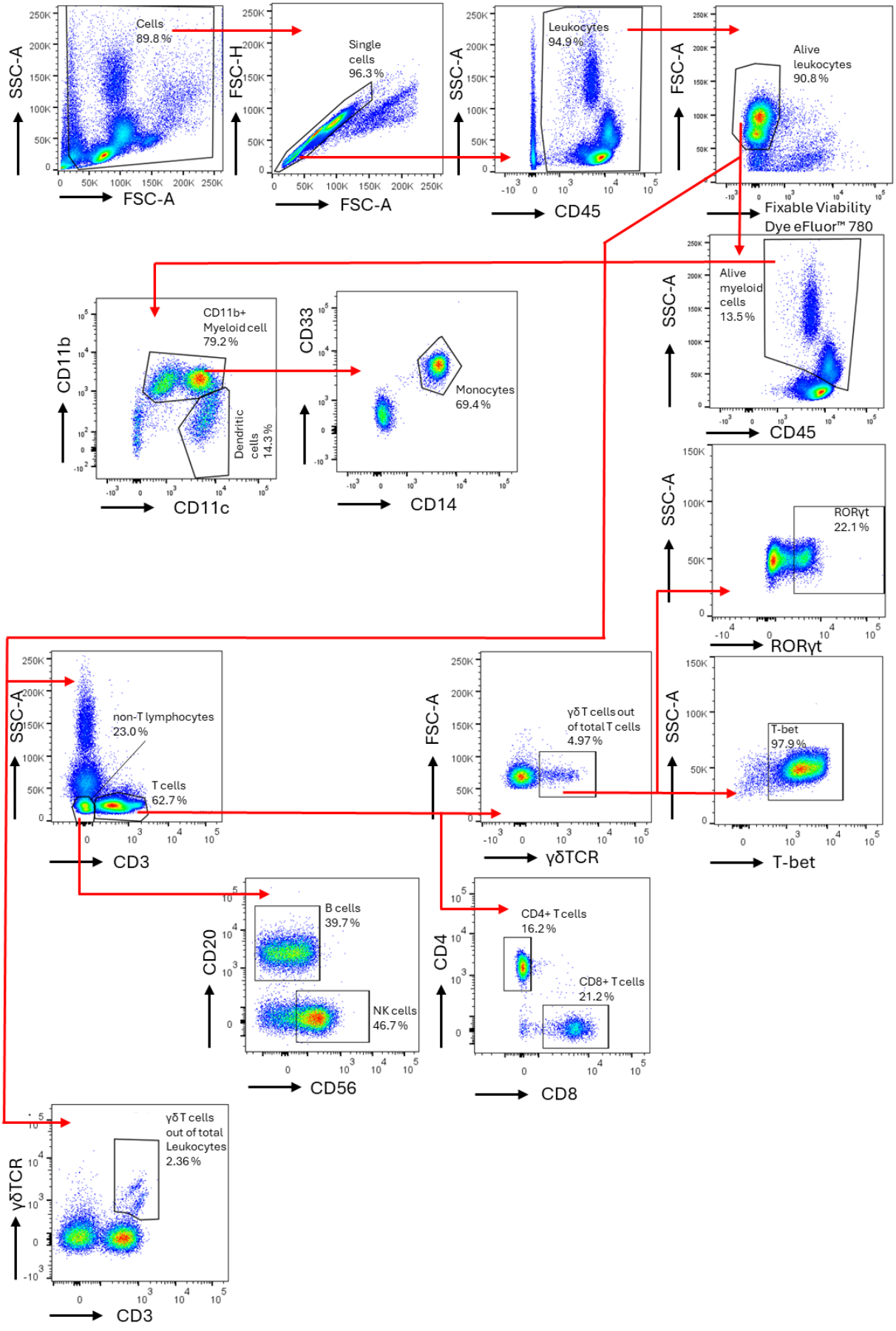

Cells
89.8 %
SSC-A
FSC-A
Single
cells
96.3 %
FSC-H
FSC-A
Leukocytes
94.9 %
SSC-A
CD45
Alive
leukocytes
90.8 %
FSC-A
Fixable Viability
Dye eFluor™ 780
CD11b+
Myeloid cell
79.2 %
Dendritic
cells
14.3 %
CD11b
CD11c
CD33
Monocytes
69.4 %
CD14
Alive
myeloid
cells
13.5 %
SSC-A
CD45
RORγt
22.1 %
SSC-A
RORγt
non-T lymphocytes
23.0 %
T cells
62.7 %
SSC-A
CD3
γδT cells out
of total T cells
4.97 %
FSC-A
γδTCR
T-bet
97.9 %
SSC-A
T-bet
B cells
39.7 %
NK cells
46.7 %
CD20
CD56
CD4+ T cells
16.2 %
CD8+ T cells
21.2 %
CD4
CD8
γδT cells
out of total
Leukocytes
2.36 %
γδTCR
CD3

Fig. S7 Gating strategy for peripheral blood mononuclear cells (PBMCs) samples. PBMCs were isolated from whole blood. Afterwards, expression of specific markers was determined by flow cytometry. Gating strategy: debris was excluded based on forward and side scatter (FSC, SSC), then doublets were excluded, leukocytes were selected based on CD45 positivity, and alive leukocytes were gated based on Fixable Viability Dye negative population. Alive myeloid cells were selected based on scatters from alive leukocytes. Dendritic cells ($CD11b^{-/dull}CD11c^{+}$) and $CD11b^{+}$ myeloid cells were selected from alive myeloid cells. Monocytes were selected from $CD11b^{+}$ myeloid cells based on CD33 and CD14 positivity. Non-T lymphocytes ($CD3^{-}$) and T cells ($CD3^{+}$) were selected from alive leukocytes based on CD3 expression. From non-T lymphocytes, B cells were determined based on the expression of CD20 and NK cells based on the expression of CD56. From T cells, helper T lymphocytes were determined based on CD4 expression, cytotoxic T cells based on CD8 and γδ T cells based on γδTCR expression. The transcription factors RORγt and T-bet were determined from γδ T cells. γδ T cells out of total Leukocytes were determined based on CD3 and TCR positivity. T cells out of total Leukocytes were determined based on CD3 and γδTCR positivity

Fig. S8

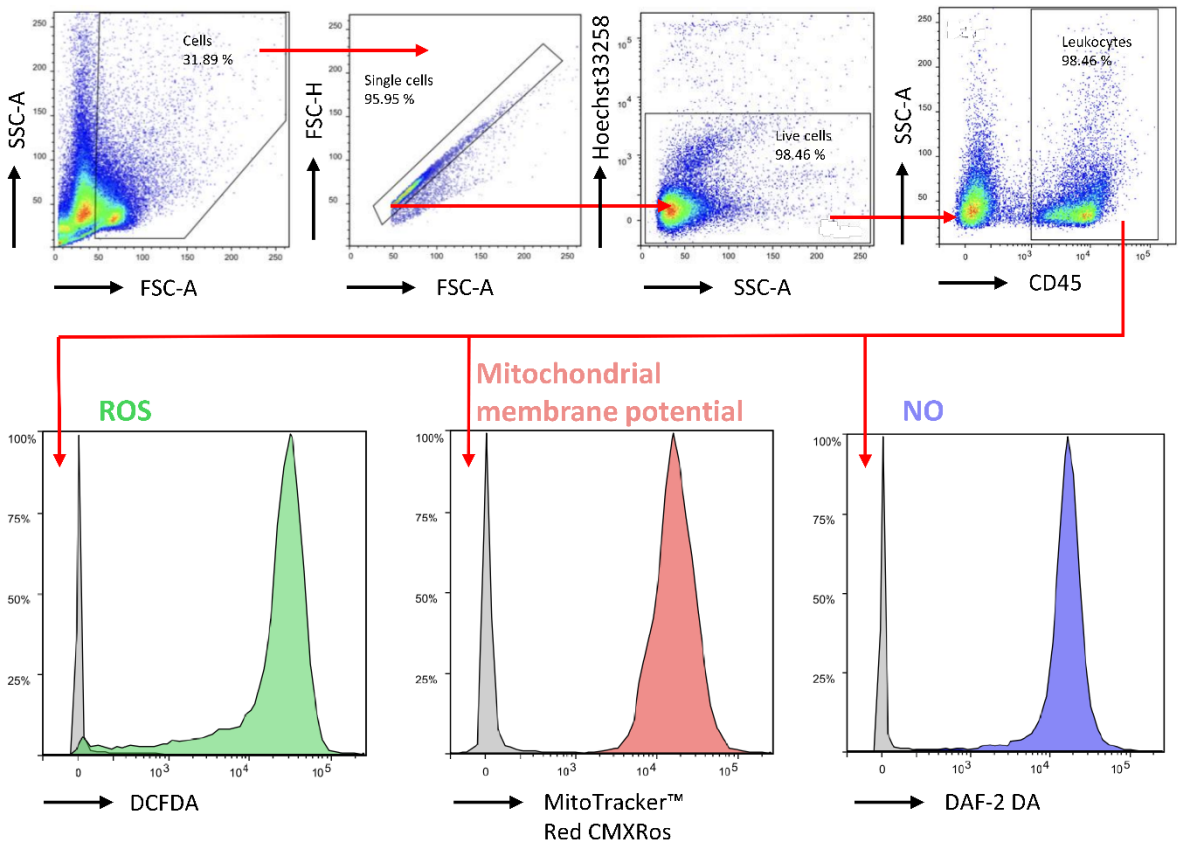


Fig. S8 Gating strategy for Reactive Oxygen Species (ROS), Membrane potential of mitochondria, and intracellular Nitric Oxide staining. A single cell suspension was prepared from the spleen. Afterwards, cells were intracellularly stained for the detection of ROS production using 2',7'-Dichlorofluorescin diacetate (DCFDA, green), for mitochondria membrane potential using MitoTracker Red CMXRos (red), and for intracellular nitric oxide (NO, blue) using diaminofluorescein-2 diacetate (DAF-2 DA). Gating strategy: debris was excluded based on forward and side scatter (FSC, SSC), then doublets were excluded and live were gated based on Hoechst 33258 negative population (live). From live leukocytes ($CD45^+$) were determined mean fluorescent intensity for DCFDA, Mitochondrial membrane potential, and DAF-2 DA. Grey – unstained FMO control

Discussion II.

The present publication focused on the comprehensive impact of cold exposure on the immune system. The study was conducted using an outbred rat model of the Wistar strain, which is characterized by its relatively high genetic diversity. This choice was motivated by the hypothesis that the results described in this study would have broader application and increased biological relevance, even if this were accompanied by a reduction in reproducibility. The utilization of a rat model, as opposed to the conventional mouse model prevalent in immunological studies, offers substantial advantages. However, this choice is counterbalanced by the constrained availability of specific reagents in contrast to the abundant availability in the case of mice. Furthermore, the accessibility and utilization of genetically modified rat strains remains comparatively constrained. However, the most fundamental advantage is the size of the rat and its physiology more similar to humans [258],[259], unlike mice, which have distinct thermoregulatory strategies [260]. As such, the rat model provides a more direct translation to human physiology and medicine.

Following verification of the experimental setup previously described by the coauthors [167], a more thorough examination of the immune aspects present within adipose tissue was performed. We examined the functionality of our model in relation to the amount of BAT, which has been identified as a marker of cold acclimatization. Our observations revealed a significant increase in BAT after long-term adaptation. Adipose tissue, a highly metabolically active organ, contains a wide range of immune cells that produce substantial amounts of cytokines [261]. In WAT, a substantial augmentation in cytokine production was observed following a one day of exposure to cold, a finding that is in alignment with the existing literature. This is attributed to the participation of adipocytes in adipose tissue remodeling after cold exposure [262]. In contrast, a shift in cytokine production within BAT was observed only in response to prolonged cold exposure, manifesting as an increase in both isoforms IL-17A and IL-17F. The activity and origin of these IL-17 cytokine isoforms are well documented in the literature in the context of adipose tissue and thermogenesis [189],[203],[263],[264]. Overall, our results suggest that WAT cytokine production is more stress-related and may play a role in activating the thermogenic function of γδ T cells within BAT. Conversely, BAT has been observed to exhibit cold-induced activation that is associated with sustained cytokine production in cold-acclimated animals.

In the attached study, we established an association between IL-17A production and γδ T cells. We conducted a more detailed examination of this particular immune cell population and identified significant differences in its representation during periods of cold exposure. This finding stands as the pivotal outcome of the study. In animals exposed to a reduced temperature for 14 days, we observed a significant increase in the number of γδ T cells in the thymus, peripheral blood, peritoneal cavity, and spleen. This heightened presence suggests an enhanced systemic production of γδ T cells, underscoring their critical role in cold acclimatization processes beyond adipose tissue. The enrichment of γδ T cells in BAT after cold

exposure was previously reported by Kohlgruber et al. [189], but no study had documented an overall systemic increase. In other immune populations, no significant changes in representation or numbers were detected. However, it should be acknowledged that due to the selected model, it was not feasible to detect all immune populations or discern differences within γδ T lymphocytes. This challenge remains unresolved and thus offers a very interesting research area. To validate our findings, we analyzed blood samples obtained from human volunteers who swim in ice water. Despite the modest sample size and considerable heterogeneity within the cohort, we confirmed an increased prevalence of γδ T cells in peripheral blood. Furthermore, we detected alterations in the transcription factors RORγt and T-bet in this population. In other immune populations, the volunteer groups did not differ. Consistent enrichment of γδ T cells in peripheral blood after long-term or repeated cold acclimatization in both outbred rats and human volunteers suggests a cross-species mechanism involved in mammalian thermogenesis.

Consequently, we conducted a more in-depth evaluation of the differences in γδ T cells induced by cold exposure. We used RNA sequencing of cell-sorted γδ T cells isolated from the rat spleen. The results revealed up-regulated gene expression associated with cation channels in γδ T cells after cold exposure, further confirming their role in thermoregulatory adaptation, which is conserved across species [265]. In cells isolated from animals exposed to one day of cold, gene expression was associated with an activated γδ T cell phenotype [266],[267], and this finding that was supported by flow cytometry analysis. Furthermore, the γδ T cells analysis obtained from long-term adapted animals revealed up-regulation of genes related to glutamate and acetylcholine responses, as well as genes associated with antigen processing. These gene groups influence critical functions in T lymphocytes, including activation, proliferation, and migration [268],[269]. Migration was further influenced by augmented expression of the semaphorin family in both groups of cold-adapted animals, supporting the established link between the sympathetic nervous system and the immune response [270],[271]. Furthermore, we confirmed the increased migratory capacity of γδ T cells by validating the main chemotactic pathway CCR6-CCL20 [272],[273], where CCR6 expression is predominantly observed in activated γδ T lymphocytes [189],[274]. CCR6 expression in γδ T lymphocytes increased significantly after cold adaptation, while CCL20 expression in the spleen of both adapted groups was suppressed. Together, these observations, along with changes in gene expression related to semaphorins, chemokines/chemokine receptors, and glutamate and cholinergic receptors, clearly indicate that cold exposure modifies the migratory capacity of γδ T cells.

Several older studies have addressed changes at the systemic level in the composition of immune populations during cold exposure. These studies have yielded mainly contradictory results. Jánský et al. described an increased trend in the total number of T and B lymphocytes in humans after cold adaptation [173]. Conversely, more recent studies have reported Tregs induction [174], while others articles have noted a decrease in Treg population and suppression of innate immunity [155],[156]. Additionally, some studies have reported an

increase in organ infiltration by macrophages and T cells after long-term cold exposure [275]. Another study revealed an increase in neutrophil percentage and changes in lymphocyte subpopulations [172]. Interestingly, the most widely studied immune cell type in response to cold stress are NK cells. In our volunteer samples examined, NK cells did not exhibit substantial alterations; however, a tendency toward a decline was evident. To achieve statistical significance while preserving adequate testing power, it would be necessary to double the volunteer sample size. The observed reduction in NK cell representation after cold exposure aligns with findings from other studies [276], which further supports the role of NE in the suppression of NK cells [277]. However, even within the context of NK cells, the existing literature presents conflicting results. Some authors have observed an increase in NK cell numbers under cold conditions [278],[279], while others did not found a change [280]. The different results can likely be attributed to different temperature models and severity of cold exposure, which contributes to the variability of immunological parameters [281]. In light of these findings, it can be concluded that there is still a lack of knowledge regarding the dynamics of immune populations during cold exposure. This underscores the necessity for further research in this area to elucidate the fundamental mechanisms of cold acclimatization.

The dynamics of cell populations in adipose tissue could also be of considerable interest. Adipose tissue is not only composed of adipocytes but also contains a variety of other cell types [282]. The immune populations present in WAT and BAT exhibit unique characteristics that contribute to the health status and physiological functions of tissues [283]–[285]. During cold exposure, adipose tissue undergoes activation, leading to the beige adipogenesis process and the initiation of thermogenesis. In this process, immune cells play a key role in establishing a suitable microenvironment, as described in the introduction to this work. However, the dynamics and representation of individual cellular immune populations remain a subject of debate. Our results, consistent with those of Kohlgruber et al., indicate a crucial role for γδ T cells [189]. However, these results are not consistent with those reported by Ye et al. in their extensive meta-study of changes in immunocytes in BAT and beige adipose tissue. They concluded that the proportions of monocytes, naïve, and memory T cells were significantly up-regulated, while the proportion of NK cells was down-regulated in BAT during cold stress. Interestingly, the proportion of γδ T cells was not affected [286]. It is important to note that the observed percentages may not necessarily reflect the total cell counts, and the significance of differences in minority populations like γδ T cells could be obscured in large-scale population analyses.

Mice lacking TLRs have been observed to exhibit altered thermogenesis [217], while TLR stimulation compromises adaptive thermogenesis [215]. Additionally, TLR activation has been associated with a decrease in UCP1 in adipocytes and suppression of mitochondrial biogenesis [218],[219]. To determine whether cold exposure affects immune system function, we induced inflammation *in vivo* by stimulating TLR2 using the synthetic ligand Pam2CSK4. The inflammatory response triggered by Pam2CSK4 application involves complex changes in

neuropeptides, cytokines, hormones, and other factors in the body, which influence immune cells [212], including γδ T lymphocytes [213].

Despite the TLR2 activation, no alterations in the outcome of thermogenic adaptation were observed. In both control and TLR2-stimulated animals, long-term cold exposure led to a significant increase in BAT weight. However, we observed significant changes in immune dynamics. Although neither cold nor Pam2CSK4 application alone affected serum cytokine levels, the combination of these effects significantly altered the entire cytokine environment. It is noteworthy that these changes did not follow a clear pro- or anti-inflammatory pattern. It is plausible that cold exposure led to the production of various factors, including neurotransmitters [285],[287], which modulated TLR sensitivity and thus resulted in an altered response to Pam2CSK4. Cold exposure has been demonstrated to activate TLR signaling pathways in fish [288], and modulate cytokine production by LPS-stimulated monocytes [289]. Our findings further support this hypothesis, as we observed altered serum NE levels, a key regulator of thermogenesis, after the combination of cold exposure and TLR2 stimulation. It is possible that the dose of Pam2CSK4 used in our study was insufficient to directly affect adaptive thermogenesis; however, it was sufficient to activate the immune system. This is evidenced by the presence of activated CD11b/c myeloid cells expressing increased TNFα levels and costimulatory molecules. However, a more precise identification of the responsible cell population was not possible due to the limitations of our model. The influence of TLR activation on immune cells is further supported by the increased presence of ROS, NO, and increased mitochondrial potential in the cold exposed groups, which were not observed in the unstimulated groups. Collectively, these observations suggest that cold exposure modifies the immune response to pathogens and affects the sensitivity of TLRs.

We also observed altered immune responses in PBMCs obtained from human volunteers exposed to Pam2CSK4 and LPS. We demonstrated that individuals who regularly practiced cold swimming did not show changes in IL-1β and IL-6 production compared to controls but had reduced IL-10 and TNFα production. In a similar study, habitual winter swimmers showed decreased LPS-induced release of IL-1β and IL-6 [176]. It is noteworthy that several studies have identified a significant association between gender and response to cold stimuli, suggesting differences in thermal and metabolic activity [290],[291], as well as in cytokine production [292]. Conversely, other studies have not found such gender-based differences [278]. The analysis of our data set did not reveal significant gender-related trends. The observed discrepancies among studies can be ascribed to various factors, including the severity of cold exposure, additional stressors such as exercise, and methodological differences. Cold acclimatization presents a substantial challenge on both the neuroendocrine and immune systems. Yet the resulting adaptation is highly individualized, influenced by personal characteristics and the specific acclimatization protocol used. Our results indicate that adaptive mechanisms occur in habitual cold swimmers, which can have varying effects on different immune cell populations and cytokine responses.

Final Discussion

The attached publications demonstrate that temperature exerts a substantial influence on the immune system. *In vitro* experiments using macrophages with the temperature-sensitive TRPV1 channel, activated by capsaicin, provided valuable insights into thermogenic stimuli. The *in vivo* model of cold acclimatization in rats further demonstrated modulation of immune cells. The validation of these findings in human volunteers underscores the relevance of our research to human biology. It is evident that *in vivo* models present a more complex system, with temperature fluctuations impacting numerous physiological and cellular processes beyond the immune system alone. While the data obtained from these experiments provide a broad understanding of temperature-induced responses, isolating specific mechanisms within the overall acclimatization process remains challenging. Conversely, the study of a specific protein can elucidate the precise cellular mechanisms; nevertheless, it is insufficient to predict the response of a tissue or an entire organism. While the first publication offers insights into the impact of TRPV1 stimulation on a particular cell type within an inflammatory environment, it is important to note that this response may vary among different immune cell types, as evidenced by previous literature [129],[131],[133],[134]. Secondly, the *in vivo* setting may exhibit various characteristics associated with the complexity of organismal systems. Neurons respond to the temperature stimuli and transmit information, in the case of a strong life-threatening stimulus, transmit the information to a designated CNS center, which rapidly processes the stimulus and disseminates thermoregulatory instructions with greater readiness than the immune system cells can register the sensation. An illustration is the human protective response to acute burns from a hot object, where TRPV1 activation of nociceptive neurons triggers an immediate neural pathway that leads to withdrawal from the heat source [293]. Whether immune cells also respond during this short interval remains uncertain. However, the role of immune cells is known if a protective response is not initiated in time and a burn occurs [294]. By studying specific mechanisms at the protein and cellular levels, we can elucidate partial aspects that are crucial for understanding overall mechanisms, which we can then observe in *in vivo* experiments or empirical life experience.

However, ambient temperature has been demonstrated to exert a significant influence on the outcomes of scientific experiments. In an *in vitro* system, the cultivation is optimized to ensure ideal conditions. However, in the context of daily life, the organism is not confined to optimal conditions and is subject to fluctuating temperatures. Culture temperature has been demonstrated to affect numerous cell characteristics, as evidenced by the example of mesenchymal stem cells (MSCs). These cells are essential in the development, regeneration, and immunomodulation of the organism [295]. Their standard culture temperature is 37 °C. However, recent studies have shown that at lower temperatures, MSCs exhibit enhanced myogenic differentiation properties, which is attributed to activation of TRPM8 channels [296]. Conversely, at higher temperatures, differentiation of MSCs towards osteogenesis is observed [297],[298]. Increased culture temperature decreased the immunomodulatory

properties of MSCs [299],[300], while reduced temperature increased their antioxidant effects [301],[302]. A similar effect of culture temperature is also documented in the literature focusing solely on immune populations [303]–[305]. Interestingly, a similar pattern of findings was observed in experimental animals.

Although humans usually operate at temperatures within their thermoneutral zone, most laboratory rodents are housed at temperatures below this border. Consequently, these animals have an increased energy requirement to generate heat. This phenomenon has been demonstrated to exert an influence on their immune system. Some studies have shown that the results obtained in animals kept in thermoneutral conditions were different from those obtained in rodents kept at normal laboratory temperatures. This casts doubt on the results observed in rodent models of human disease [306]. Housing at a thermoneutral temperature has been observed to exert protective effects in relation to bacterial[307],[308], viral [309] and parasitic infections [310]. The authors also observed different results in cancer [311], metabolic diseases [312],[313], Alzheimer's disease [314], respiratory disease [315], and graft vs. host disease [316]. On the other hand, studies have been conducted that question the importance of thermoneutral housing of animals. They argue that the thermoregulatory curves of humans and animals housed in conventional laboratory conditions are metabolically more similar than those of animals housed at excessively elevated temperatures [317]. Nevertheless, it is beneficial to contextualize these findings within the specific temperature parameters of the experimental setup. A comparative analysis with data obtained at conventional laboratory temperatures might provide more detailed insights [306]. The findings of our publications are thus constrained by this factor. However, it is important to note that the animals utilized in our cold acclimatization model were male Wistar rats, which are susceptible to vascular dysfunction and altered metabolic function in a thermoneutral environment [318]. Consequently, we assert that the temperatures used in our experiments were carefully selected to align with the experimental model.

However, when evaluating the results of thermoregulation experiments, it is important to consider the variability among individuals within the population. The individual capacity to thermoregulation is influenced by a multitude of factors, including sex, age, height, weight, time of day, physical condition and geographical location [319],[320]. Traditional East Asian medicine employs a classification system based on the concept of cold-heat patterns. This concept is associated with the Yin and Yang energies in the body, with a cold pattern being related to an excess of Yin and Yang deficiency, while a heat pattern reflects excess Yang and Yin deficiency [321]. The significance of these patterns has been verified in scientific articles, which have identified them as useful prognostic markers [322],[323], as well as for elucidating the differential effectiveness of treatments [324]. An in-depth explanation of this concept was provided by Kim et al. in their comprehensive meta-study. They evaluated genes related to thermoregulation and compared them in different thermosensation diseases. Therefore, they revealed a strong association between these genes and gene set linked to inflammatory mediator regulation of TRP channels. They describe TRP channels as crucial factors in the role

of thermoregulation and as the regulators of the immune system, due to their ability to modulate inflammatory mediators in response to temperature changes, thereby elucidating key mechanisms underlying individual differences in cold and heat patterns [325].

There is no doubt about the close connection between the nervous and immune systems in thermoregulation. In our studies, we have brought new insights that contribute to the overall understanding of this complex network of processes. In the context of TRPV1, the channel has been shown to play an important role in both neuroimmune interactions. However, in accordance with the published literature, our findings have revealed that its modulation also has a significant impact on immune cells [70],[129],[131],[133],[134]. In our cold exposure study, we focused primarily on immune aspects; however, the observed changes in NE levels in TLR-stimulated acclimatized animals were noteworthy. It is well established that cold adaptation processes are mediated by adrenergic signaling and the release of relevant neurotransmitters [24],[326]. Immune cells respond to neurotransmitters through their ARs and thus play a role in the broader thermoregulatory process [327]. In addition, immune cells have been shown to induce beige adipocytes even in the absence of the sympathetic nervous system [197], highlighting the critical role of the immune system in this process.

It has been shown that β3-AR stimulation alone induces BAT, although it primarily triggers gene expression that is distinct from that of whole-body cold acclimatization. The research revealed that the differences were mainly in metabolic glycolytic genes and genes involved in fatty acid processing. However, genes associated with vascularization and inflammation of adipose tissue remained unaltered [328]. Consequently, it can be concluded that neural signaling plays a unique role in the regulation of immune cells in cold exposure. This hypothesis is further supported by the γδ T cell RNA sequencing, which revealed a significant alteration in the expression of a gene set associated with inorganic cation transport (including TRP channels) and a shift in neurotransmitter signaling from acute ATP release to a complex interplay between different neurotransmitters. These results indicate that changes in neurotransmitter levels during cold acclimatization closely mirror the regulation of their respective receptor genes. The influence of γδ T cells may be direct, given from their proximity to neurons, where they have been shown to induce IL-17A secretion and regulate pathways associated with inflammation and metabolism, as described in recent studies employing a rosacea model [329]. Additionally, our analysis identified a set of genes linked to semaphorins, which have been identified as critical molecules exhibiting differential expression during cold exposure. Semaphorins are proteins that connect the sympathetic nervous system with the immune system [271]. Moreover, they have been documented in BAT, where they have positive or negative effects on its activation [193],[271],[330],[331]. In the immune system, they play a role in different phases of the immune response, including migration, proliferation, and activation [270]. Furthermore, certain semaphorins have been reported to be significantly associated with temperature sensitivity [334]. Therefore, it can be hypothesized that this could be a bridge between the nervous system and the immune response to changes in ambient temperature.

Ambient temperature influences the nervous system, metabolic activity, and immune system, thereby affecting susceptibility to infections and the ability to combat autoimmune diseases and cancer [157],[168],[169]. From a clinical perspective, it would be advantageous to modulate the immune response by setting the optimal temperature. This objective has been facilitated by understanding temperature-dependent receptors and their specific ligands. A notable example is the use of capsaicin patches, which are designed to sensitize TRPV1, in the treatment of peripheral neuropathic pain [332]. A comparable approach is employed in menthol ointments, which have been demonstrated to activate TRPM8, yielding a similar therapeutic effect [333]. Given the results obtained, it can be assumed that by choosing the appropriate ligand concentration or temperature, it might be possible to modulate the phenotype of macrophages through TRP channels and subsequently attenuate the acute inflammatory response. Nevertheless, current biomedical ideas are even more ambitious. Recent advances have enabled the engineering of macrophages that possess an integrated heat-inducible genetic switch enabling autopolarization towards the inflammatory M1 phenotype, while simultaneously repolarizing TAMs within the tumor environment. Applying external heating, the authors triggered gene expression in these modified macrophages, leading to their direct polarization and release of immunomodulatory factors. This mechanism also validated in mouse transplantation models [334]. This finding suggests that temperature may serve not only as a therapeutic target but also as an intriguing bioengineering tool.

The study of ambient temperature and the comprehension of its multifaceted effects on living organisms remains a prominent area of research. There is still ongoing research aimed at elucidating the fundamental mechanisms by which immune cells respond to temperature changes. While the intricate relationship between temperature and immune cell activation is not yet fully understood, it is well established that immune cells play a critical role in thermoregulatory pathways. Currently, temperature stimuli are considered a promising therapeutic and engineering instrument in clinical settings. In this dissertation, my aim was to contribute novel insights to the field of thermoregulatory processes and the impact of temperature on immune cells. This thesis elucidates the role of the temperature-dependent TRPV1 channel on macrophages in LPS-induced inflammation and describes fundamental systemic changes during cold acclimatization, which are primarily associated with the population of γδ T lymphocytes and TLR2-mediated inflammation. This work offers a unique connection between thermogenic stimuli and the immune system, as well as the inflammatory environment.

Conclusions

Aim 1) To elucidate the impact of activation of the high-temperature-dependent TRPV1 channel in macrophages

We confirmed TRPV1 expression in macrophages upon capsaicin stimulation in inflammatory environment. However, stimulation with capsaicin alone, in the absence of an inflammatory stimulus, did not elicit substantial changes (**1b**). In inflammatory conditions, capsaicin stimulation triggered calcium influx into the cell and phosphorylation of ERK1/2 though without nuclear translocation (**1a**). Notably, stimulation of LPS-pretreated macrophages with capsaicin resulted in a switch from an inflammatory M1 phenotype to an anti-inflammatory M2b-like phenotype. This shift was characterized by up-regulation of costimulatory molecules and MHC, and was confirmed by the promotion of proliferation of CD4+ T lymphocytes in a co-culture assay. A concomitant decrease in inflammatory cytokine expression was also observed (**1c**). Furthermore, these M2b-like macrophages demonstrated an increased susceptibility to infection by the parasite *Leishmania mexicana*.

Aim 2) To elucidate the alterations in the immune system in response to cold exposure

A comprehensive examination of the immune system in rats during the cold acclimatization process revealed substantial changes. Dynamic alteration in cytokine production was detected in both WAT and BAT. In addition, an increase in the presence of γδ T lymphocytes was observed in peripheral blood, thymus, spleen, and peritoneal cavity, thereby confirming their systemic enrichment after cold exposure. However, no significant changes were detected in other populations of immune cells (**2a**). Consequently, we have postulated that γδ T cells may function as critical regulators in the thermogenesis induction. Under physiological cold exposure, the most significant changes in gene expression in γδ T cells were observed in genes responding to nerve signals. Specifically, we identified a set related to inorganic cation transport and a gene family of semaphorins. This finding suggests that the neuroimmune axis plays a crucial role in the thermogenic response (**2b**). Moreover, an increase in γδ T cell population was observed in the peripheral blood of volunteers who regularly bathe practice cold-water immersion. Furthermore, we observed an expression of key transcription factors, particularly T-bet and RORγt, suggesting potential alterations in γδ T cell function under prolonged cold exposure (**2c**).

After inflammatory activation of TLR2 in cold-exposed animals, BAT weight was not affected. This suggests that maintaining temperature homeostasis is a priority for the organism. However, the immune response to TLR2 stimulation in cold-exposed animals deviated from

that observed in animals housed at laboratory temperature. The immune response was predominantly mediated by the myeloid population CD11b/c and was characterized by altered levels of cytokines and NE in serum, along with increased ROS, NO, and mitochondrial potential of immune cells (**2d**). In PBMC samples obtained from human volunteers, we observed a reduced production of TNFα and IL-6 after LPS stimulation in cold swimmers compared to the control group.

This work provides novel insights into the study of thermogenic stimuli and the immune response in the context of the temperature-dependent TRPV1 channel in macrophages, as well as in the context of whole-body cold acclimatization. Our findings enrich basic knowledge and offer a significant therapeutic opportunity, further strengthening the contributions of this dissertation. In this dissertation thesis, I have successfully achieved my objectives, as evidenced by the comprehensive results that demonstrate a substantial impact of thermogenic stimuli on the immune system and underscore the importance of temperature-related research.

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